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CHANGES IN THYMUS AND LYMPH NODE ACTIVITY, AND ALTERATIONS IN BONE MARROW LYMPHOCYTE LEVELS DURING RECOVERY OF THE GUINEA-PIG FROM WHOLE BODY GAMMA IRRADIATION

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It has been shown by a quantitative technique (Harris, Herdan, Ancill and Yoffey, 1954) that lymphocytes form an important group amongst the marrow nucleated cells of the normal young guinea-pig. In a later series approximately 25 per cent of the total nucleated cells in the marrow were lymphocytes (Harris and Harris, 1956, unpublished).

From these normal findings it was thought that quantitative study of the various marrow cell groups at close intervals during recovery from whole body irradiation might reveal significant changes in the marrow lymphocyte population if these cells have an important role in haemopoiesis. The pattern of recovery in the main cell groups and their temporal relationships could also be studied. A preliminary note on this subject has been published (Harris, 1956) and further experiments have substantiated these results.

The purpose of this paper is to draw attention to 3 features emerging from the experiments. Firstly, that up to the 18th day after irradiation changes in thymic weight, which histologically appeared to be due to a variation in lymphocyte content of the cortex, can be statistically correlated with changes in marrow lymphocyte levels. Thus during the first 18 days after irradiation the increase in the bone marrow lymphocytes occurred concurrently with their increase in the cortex of the thymus. The significance of this will be considered. Secondly, that although the lymphocyte is very sensitive to irradiation, recovery of lymphocytopoiesis in the thymus and mesenteric lymph node, following the dose of irradiation used in these experiments was relatively rapid. This recovery and the restoration of marrow lymphocyte levels preceded the recovery of granulocyte and erythroid cells in the marrow. Thirdly, that the blood lymphocyte levels occurring during recovery from irradiation were not necessarily good indices of the underlying state of activity in the lymphoid tissues examined, particularly the thymus.

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MATERIAL AND METHODS

Young male guinea-pigs of the Dunklin-Hartley strain were used in this study. At least 7 days before irradiation a preliminary blood investigation was made using blood obtained from an ear vein. Total red and white cell counts, differential white cell count, reticulocyte count and haemoglobin estimation were made.

On reaching the weight of 400 g. each animal received 150–170 r whole body irradiation using a Cobalt-60 unit as source. This dose is slightly less than that described by Ellinger (1945) as the $L.D._{50}$ for guinea-pigs, although differences in sensitivity occur with strain of animal. Irradiation was under general anaesthesia (intraperitoneal sodium pentobarbitone) to ensure constant position and hence uniform dosage, the animal being closely confined in a wooden-framed cage covered by a wide-meshed string net.

A second blood investigation was made immediately before quantitative examination of the bone marrow using the technique described in a previous communication from this Department (Yoffey, 1956). Marrow smears, fixed in methyl alcohol, were stained with MacNeal's Tetrachrome stain. At least 500 cells were counted upon each of 2 smears in every experiment. Femoral marrow, spleen, thymus and mesenteric lymph node (Pancreas Aselli) were taken for histological examination. Tissues were fixed in Zenker-Formol, embedded in Ester wax and sectioned at 3 μ. Sections were stained with haematoxylin, eosin azure II and with toluidine blue and eosin orange G, using the method of Dominici (1902).

Lymphocytopoietic activity during recovery was estimated by histological examination of lymph node and thymus and by recording weight changes in the thymus. Activity in comparable sections from lymph nodes at various intervals during recovery was assessed histologically by the size, number, cell content and morphology of lymphoid follicles : the extent of diffuse lymphoid tissue and mitoses in it : the cell content of various lymph channels in the node, namely in the medulla, in the hilar region and especially that part of the cortical sinus related to the hilum. Thymus activity was assessed by the thickness of the cortex relative to the medulla and the incidence of mitotic figures in both zones. However, the assessment of activity in lymphoid tissues by histological appearance alone, in the absence of other evidence, *e.g.*, biochemical, requires caution. For example, cyclical activity has been described in lymph nodes (Conway, 1937). Variation in lymph and blood flow through lymphoid tissues, with consequent variation in their lymphocyte content, are important factors which may, in part, determine the histological picture at any particular time. Nevertheless, despite these limitations, definite histological changes in the thymus and lymph node were detected at the various stages of recovery.

Weight changes were recorded as an index of activity in the thymus which is easily identified and may be accurately removed in the young guinea-pig : the wet weight was recorded immediately after removal. The weight of the Pancreas Aselli was not recorded due to the gland's variable size.

Thoracic duct lymphocyte output during recovery from irradiation also provides evidence on lymphocytopoietic activity since it represents some of the lymphocytes entering the blood stream indirectly (cf. those entering directly via blood vessels of lymphoid tissue). Reference will be made to preliminary experiments in which thoracic duct lymphocyte output was determined after the same dose of irradiation as used in these experiments.

RESULTS

The results are based upon material from 118 experiments. The observation periods after irradiation and the number of experiments in each period is shown in the table. Control data were obtained from 18 animals subjected to the same conditions as those in the experimental group, except for actual irradiation. These animals were examined at the same intervals as the experimental animals.

Marked changes occurred in the haemopoietic organs of this strain of guinea-pig following the above dose of irradiation. At the same time the mortality rate was low. Recovery of the marrow appeared to be complete by the end of the 5th week after irradiation.

TABLE.—*Distribution of Observation Periods After Irradiation and Number of Experiments in Each Period*

	Hr.	Days																
Time after irradiation	4	1	2	3	4	6	8	10	12	14	16	18	20	22	24	26	28	35
Number of experiments	8	5	5	6	6	7	7	8	8	5	5	5	7	8	8	8	5	7

Statistical analysis of data

The means and their standard errors were calculated for the thymic weights and bone marrow lymphocyte levels in all the observation periods. Student's "*t*" test was applied to determine whether the difference of certain means was significant. The coefficient of correlation was calculated between thymic weight and bone marrow lymphocyte content for the total observations and over certain periods.

Changes in thymic weight

The weight changes are shown graphically in Fig. 1. After an initial decline in thymic weight, recovery occurred and normal levels were reached by the 18th

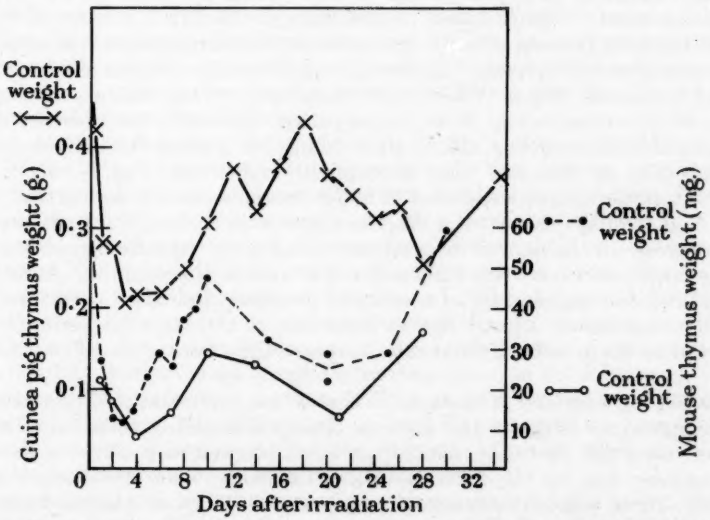


FIG. 1.—Weight changes in thymus of guinea-pig and mouse after whole body irradiation.

day. This recovery was only temporary, being followed by a second decline in weight which reached its lowest level on the 28th day. Not until the 35th day did thymic weight return once more to normal levels. Even 4 hr. after irradiation there was some decrease in weight but it is not statistically significant. The weight loss was well marked by 24 hrs. The minimum weight occurred on the 3rd day when it was less than 50 per cent of the controls, the difference being

statistically significant ($p = < 0{\cdot}001$). The weight remained low until the 6th day when recovery began. It gradually increased to reach a peak on the 18th day when it was within normal limits (no statistically significant difference from controls). There now followed another statistically significant decrease in weight ($p = < 0{\cdot}01$), reaching a second low level on the 28th day. By the 35th day the thymic weight had again returned almost to normal.

Thymus histology

Well defined histological changes occurred. The initial phase of injury and cell depletion was followed from the 6th day by a gradual restoration of thymic structure, so that sections of tissues taken between 12 and 20 days after irradiation were comparable with those from controls. This recovery was only temporary and was followed by a decrease in lymphocyte content, the decline being most evident by the 28th day. Finally, examination of sections 35 days after irradiation showed that there was, once more, restoration to normal appearance.

The most conspicuous features after 4 hr. were pyknosis and cell debris, especially in the cortex (Fig. 3), but there was no definite evidence of cortical thinning. Cortical depletion was detectable by the 1st day (Fig. 4) and much of the debris had disappeared, but there was still evidence of phagocytosis. On the 2nd day debris had virtually disappeared and cortical thinning was now obvious. There was a characteristic " moth-eaten " appearance of the cortex in these early stages (Fig. 5) due to 2 factors. Firstly, the presence of macrophages, the appearance corresponding to the " pitting " in the thymus following injection of adrenotrophic hormone in rabbits, Fig. 3 (White and Dougherty, 1946), which produces much nuclear debris, subsequently to be phagocytosed. Secondly, the presence of blood capillaries of various calibre, the smallest being 2 or 3 times the diameter of a red blood cell (Fig. 6). Some of them contained lymphocytes (Fig. 7). Even on the 2nd day, mitotic figures were seen in larger lymphocytes of the cortex. During the 3rd and 4th days there was a marked decrease in cortical thickness associated with a decrease in the number of lymphocytes (Fig. 8). Mitotic figures were quite frequent in the cortex at this time and a few were in the medulla. After the 6th day the thickness and density of the cortex gradually increased as its lymphocyte population increased. Mitotic figures were seen in the large lymphocytes of the cortex, but as the density of the cortex increased they were more difficult to distinguish.

Between the 12th and 20th days the cortex was thick and well populated with small lymphocytes (Fig. 9), the sections closely resembling those from controls. However, after the 20th day, the cortex again showed a decrease in its density and thickness, and by the 28th day its cell numbers were obviously decreased (Fig. 10). There was no evidence of increased cell debris or phagocytosis during this decline of the cortex. Final examination of the thymus on the 35th day after irradiation showed a normal appearance. The cortex had again increased in thickness and density.

Granulocytopoiesis in the thymus.—There was evidence of granulocytopoiesis in the thymus of several animals on the 26th and 28th day after irradiation. The cells were mostly eosinophils but occasional neutrophils were seen. The granulocytes occurred chiefly in 2 sites. Firstly, as groups in the interlobular connective tissue, being frequently adjacent to arteries or veins, and secondly, as groups in the cortex at the surface of the lobule. Isolated cells were present in the cortex

and to a less extent in the medulla. There were all stages of granulocyte from large blast cells, with a few azure granules, to mature polymorphs. Undoubtedly some of these mature cells could have migrated from the lumen of thin-walled blood vessels. Mitotic figures were seen in the immature granulocytes (Fig. 11). Some granulocytes had a small round nucleus resembling that of a lymphocyte, but care is required in their interpretation since the actual point of section of the nucleus determines its morphology (Shridde, quoted by Downey, 1948). In sections from one animal, 28 days after irradiation, large blast-type cells were seen at the edge of the cortex giving it an epithelial-like appearance.

Lymph node histology

After the period of injury there was a quiescent phase but from the 6th day there was increasing evidence of recovery. The recovery pattern differed from that of the thymus in that there was no convincing histological evidence of a marked decline in activity after the 18th day. Rather, there was a gradual and progressive return to normal with most of the recovery achieved by the 18th day.

As in the thymus, there was evidence of considerable cell damage 4 hr. after irradiation. Cell debris and pyknosis were particularly evident in the follicles, some debris already being engulfed by macrophages. In many follicles there was either only a thin rim of small lymphocytes, many of which were pyknotic, or it was completely absent. Consequently many follicles had a characteristic pallor and were seen to contain numbers of large cells with large pale nuclei. In some of these cells the cytoplasm was basophilic but in others it was not. They appeared to be lymphoblasts and reticular cells.

Much of the debris had disappeared by 24 hr. and most follicles consisted chiefly of the larger, more immature type of cell. The various lymph channels were conspicuous due to the absence of free cells within them. A phase of inactivity and depletion continued for several days after irradiation (Fig. 12), although some mitotic figures were seen in sections from nodes taken on the 3rd and 4th days. From the 6th day there was increasing evidence of recovery. Mitotic figures occurred in the large cells of follicles and diffuse lymphoid tissue. Large cells with basophilic cytoplasm appeared in the lymph channels of the node, especially in the intermediary sinuses of the medulla, and frequently showed mitotic figures. Medium-sized lymphocytes reappeared in the follicles.

By the 10th day, recovery, both in follicles and diffuse tissue, appeared to be firmly established (Fig. 13). Mitotic figures were frequent and the lymph channels contained numbers of small lymphocytes. Sections of nodes taken at 12, 14 and 16 days showed clear evidence of recovery (Fig. 14).

In some cases, which had shown high marrow lymphocyte levels, there was evidence of marked activity in the nodes. By the 18th day (Fig. 15) the sections were comparable with those from controls. The pattern of recovery in the later phase differed from that of the thymus. Apart from evidence of a decrease in the amount of diffuse lymphoid tissue in only one period of observation, namely at the 28th day, there was no convincing histological evidence of a marked decline in activity after the 18th day. Sections from nodes removed on the 26th day showed numerous well-formed follicles and extensive areas of diffuse lymphoid tissue. Sections of nodes from animals 35 days after irradiation were comparable with those from controls.

Thoracic duct lymphocyte output after irradiation

In preliminary experiments Hanks and Yoffey (1957, personal communication) found a marked decline in the thoracic duct lymphocyte output of 2 guinea-pigs up to 4 days, after a dose of irradiation as used in these experiments. However, more data will be required before any definite observations can be made.

Changes in bone marrow lymphocytes

It is well known that lymphocytes are very sensitive to irradiation and by 4 hours their level in the marrow had declined to 50 per cent of that of the controls (the difference being statistically significant, $p = < 0{\cdot}001$). Bone marrow smears and sections after 4 hr. showed obvious cell debris, some of which had already been taken up by macrophages. Quantitative estimations of the main cell groups showed that the greatest depression had occurred in the lymphocytes and undoubtedly these cells contribute to much of the debris seen after 4 hr. Sections of thymus and lymph node examined at this stage showed marked evidence of cell destruction. Thus the marrow cells classified as lymphocytes in these experiments seem similar in radiosensitivity to the lymphocytes of thymus and lymph node.

The number of marrow lymphocytes decreased to a minimum on the 2nd day when they were 25 per cent of the control level, the difference being statistically significant ($p = < 0{\cdot}001$).

They showed a gradual increase from the 2nd to the 10th day when the control level was exceeded. At 14 days after irradiation the bone marrow picture was quite extraordinary in that, on an average, over 60 per cent of the cells in the smears were either lymphocytes or showed a morphology transitional between that of a lymphocyte and a blast cell (Yoffey and Courtice, 1956). Bone marrow sections at this stage showed large numbers of small round cells (Fig. 16 and 17). A maximum level, well above normal and statistically significant ($p = 0{\cdot}05$), occurred on the 16th day. A second and marked decline in lymphocytes followed and continued until the 24th day when their numbers were approximately 25 per cent of the controls, the difference being statistically significant ($p = < 0{\cdot}001$). A recovery to normal levels had been made by about the 28th day after irradiation.

DISCUSSION

Changes in the thymus following irradiation

The weight changes in the guinea-pig thymus resemble the findings of Brecher, Endicott, Gump and Brawner (1949) and of Kaplan and Brown (1957) who examined mouse thymus after whole body irradiation (Fig. 1). In the guinea-pig, as in the mouse, the second decline in weight following the peak is not as great as the initial decline occurring during the first 3 days after irradiation. Lavik, Harrington and Buckaloo (1950) studied the effects of a single $L.D._{50}$ dose of ^{32}P on various phosphorous fractions of the thymus in the young adult rat. A marked decrease in D.N.A. concentration together with a 62 per cent reduction in gland weight occurred by the end of the 3rd day after injection.

What is the explanation of the weight changes? There was no evidence of inanition in the experimental animals and a graph of body-weight changes showed a steady, if somewhat subnormal, rate of increase up to 35 days after irradiation. There was no evidence of a decline in body weight to account for atrophy of lymphoid

tissue between the 18th and 28th days. The changes could have been due to haemorrhages into the thymus, fatty infiltration, or oedema, but none of these was seen in the sections examined. These weight changes can be explained by variations in the lymphocyte content of the thymus at the various stages in recovery from irradiation as shown histologically by variations in the width and density of the cortex. Comparison of the thickness of the cortex in lobules of comparable size, at various times after irradiation, showed that these changes were not chance and due to differential sectioning of lobules.

Kaplan and Brown (1957) noted that in the mouse thymus after 300 r, cell debris was almost entirely removed by the 2nd day. Between the 3rd and 4th days the cortex was repopulated by large, immature lymphoid cells. Mitotic figures, initially abundant, became more sparse. From the 4th day smaller lymphocytes replaced the larger ones. By the 15th to 20th days the cortex and medulla appeared almost normal. These authors, like Brecher *et al.* (1948), noted an abortive increase in weight but do not explain it. The second decline in weight was not associated with histological evidence of fresh cellular radiation damage.

Warren, MacMillan and Dixon (1950) investigated the effect of ^{32}P on adult mice. After a dose of 250 μc. ($L.D._{50}$, 20 days) there was an estimated 90 per cent reduction in thymic lymphocytes during the 2nd to 5th day after injection ; which was the period of maximum injury. By the 5th day the cortex was paler than the medulla, which the authors note is the " inversion pattern " described by Bloom, and had an epithelial-like structure. Following an initial reduction in size by about 50 per cent it had returned to normal by the 10th day. Lymphocytopoiesis recommenced from the 5th to the 10th day and mitotic figures were seen in the medium sized cortical cells. It appeared to be normal between the 10th and 15th days after injection of ^{32}P. From the 15th to 20th days there was hypertrophy of the thymus, sections of which showed a wide, darkly-staining cortex, packed with cells, encroaching upon the medulla and diminishing its size. At this time the size of the thymus was up to $1\frac{1}{2}$ times normal. The thickness of the cortex was about twice that of the medulla. The reaction of the thymus to this dose of ^{32}P showed that it was more sensitive than the lymph nodes and spleen, in which maximum injury occurred several days later. In view of its rapid recovery the thymus was termed " radioresilient ".

Downey (1948) studied the thymus of a young rabbit 6 days after its local irradiation (500 r). The boundary between cortex and medulla was difficult to determine and in most lobules the cortex was very narrow, but active regeneration was occurring. Many of the cells in the outer zone of the lobule were of the large blast type. Mitoses were especially frequent in this region which also contained cells with a morphology intermediate between that of a lymphocyte and a fixed reticular cell. In recovery from irradiation, lymphocytes were produced by mitosis in existing large cortical lymphocytes and by heteroplastic formation from the mesenchymatous reticulum.

Granulocytopoiesis in the thymus.—Badertscher (1920) studied the thymus of post-natal pigs for evidence of granulocytopoiesis and erythropoiesis. All stages of eosinophils were seen from mononuclear forms, resembling eosinophilic myelocytes in bone marrow, to polymorphonuclear cells. They were either in groups or as isolated cells in the lobules and interlobular connective tissue. Groups tended to be in the medulla adjacent to the cortex and single cells in the cortex. The granu-

locytes were often close to blood vessels, especially those in the interlobular connective tissue septa, and some were in mitosis. Badertscher regarded the thymus as a definite source of eosinophils in the pig : they arose from lymphocytes and by mitosis in pre-existing mononuclear eosinophils. Basophils were seen in some sections but there was no evidence for the formation of neutrophils. Reviewing previous work on this subject, Badertscher includes the work of Weill on adult rat and human thymus, who noted that eosinophils were confined to the cortex and interlobular connective tissue septa. Some of them were identical with eosinophil myelocytes of the marrow. Some were thought to arise from thymocytes and others by division of existing eosinophils. In man the thymus was a source of all types of granular leucocytes including neutrophils. Downey (1948) studying sections and imprints from human and rabbit thymus, described two types of heterophil myelocyte in the rabbit : one resembled that seen in the bone marrow and the other had a lymphocyte type of nucleus. All the thymus eosinophils appeared to arise from small and medium-sized lymphocytes. These granulocytes were most numerous in the outer part of the cortex, close to the capsule, being either scattered or in compact groups. Some were in the medulla and others around large blood vessels or in connective tissue strands. Some heterophil myelocytes appeared to develop from the reticular stroma beneath the capsule, and were thought to arise from mesenchyme. Downey described all three types of granular myelocyte in the thymus of pre-natal and early post-natal human material.

Previous workers have derived thymus myelocytes from thymic lymphocytes or from reticular cells in the stroma. However, although only observed in one animal 16 days after irradiation, typical large promyelocytes with azure granules were seen in the blood. Thus haematogenous population of the thymus by promyelocytes from the marrow may account for some of the granulocytopoiesis observed in the thymus after irradiation.

Changes in the mesenteric lymph node following irradiation

Essentially there was a gradual and progressive return to normal after the phase of injury. From 12 to 16 days after irradiation, when marrow lymphocytes were high, lymphocytopoiesis seemed well established in the nodes and in some appeared quite intense. The sections were comparable with those from controls by the 18th day. There was no convincing histological evidence of an " abortive " peak of activity as in the thymus, although 28 days after irradiation diffuse lymphoid tissue did appear to be decreased.

Brecher *et al.* (1948) found weight changes in the submaxillary lymph node of the mouse similar to those in the thymus. Normal weight was reached by the 10th day after irradiation and a peak level, above normal, on the 14th day. It became subnormal by the 21st day, not returning to normal until the 28th day. These workers thought lymphocytopoiesis in various lymphoid organs appeared normal or even above normal between 7 to 14 days after irradiation. Kaplan and Brown (1957) recorded weight changes in axillary and inguinal lymph nodes of mice after whole body irradiation. Their findings differ from those of Brecher *et al.*, since weight recovery was slow and in contrast to the thymus did not show an abortive peak. Warren *et al.* (1950), commented that the rapid recovery of the thymus contrasted markedly with the slow recovery of lymphocytopoiesis in the lymph

nodes which did not commence until 15 to 20 days after injection of ^{32}P. Even 50 days after injection there was only partial return to a scattered type of lymphocyte formation.

Thoracic duct lymphocyte output after whole body irradiation

Hanks and Yoffey found a marked fall in thoracic duct lymphocyte output of 2 guinea-pigs up to 4 days after 150–170 r whole body irradiation. Keohane and Metcalf (1958) examined the thoracic duct lymphocyte output in rats following a large dose of whole body irradiation well above the $L.D._{50}$. Output figures were obtained from groups of 5 examined 2 hr., 1 day, 3 days and 5 days after irradiation. Only 2 hr. after irradiation the output had fallen to about 14 per cent of the controls and was even lower up to 5 days after irradiation. Valentine, Craddock and Lawrence (1949) studied the thoracic duct lymphocyte output in cats for several hours after a very large dose of whole-body irradiation. There was a depression in output even 2 hr. after irradiation and it was very marked by 6 hr. No observations were made beyond 8 hr.

Changes in bone marrow Lymphocyte population

The recovery pattern of marrow lymphocytes in guinea-pig bone marrow closely resembles the findings of Brecher *et al.*, in the mouse. They noted an abrupt temporary rise in marrow lymphocytes on the 10th day, some of which were large cells with dark blue cytoplasm and fine nuclear chromatin. These were thought equivalent to the lymphoidocyte of Pappenheim and to be stem cells of the granulocytic series, since they preceded a rapid increase in myelopoiesis. These large cells described by Brecher correspond to the transitional cells described by Yoffey in the guinea-pig, and such cells were numerous in smears at the times of high lymphocyte levels in the marrow. They can be readily traced in close stages of transition to the typical myeloblast, and the marked decline in marrow lymphocytes accompanying the increase in granulocytes is most striking, Fig. 2 (Harris, 1956).

Oughterson and Warren (1956) reviewed the bone marrow findings in human victims of the atomic bomb explosions in Japan. Specimens were obtained at various periods after exposure. Lymphocytes were often noted in material obtained during the first few weeks of recovery. Almost all the samples of reticular marrow obtained when the symptoms of radiation injury were most pronounced contained large numbers of lymphocytes. From 3 to 5 weeks after exposure the average percentage of lymphocytes in differential counts was higher than that of any other cell type, *viz.* 28·6 per cent in Hiroshima and 40·2 per cent in Nagasaki. In individual cases there were levels as high as 80 per cent. Most of these cells appeared to be normal small lymphocytes, but in many cases there was a variable number of large atypical forms resembling neutrophil myelocytes or blood monocytes.

Correlation between thymic weight and bone marrow lymphocyte content

Graphical comparison of bone marrow lymphocyte levels and thymus weights shows a close similarity of the curves (Fig. 2). In plotting the marrow lymphocytes, cells classified as " transitional " were not included.

Calculation of the coefficient of correlation between thymus weight and bone marrow lymphocytes for the total number of observations (118) gives $+0{\cdot}308$ which is statistically significant ($p = 0{\cdot}001$). From the 3rd to the 18th day the correlation coefficient is $+0{\cdot}676$, which is significant ($p = < 0{\cdot}001$) and from

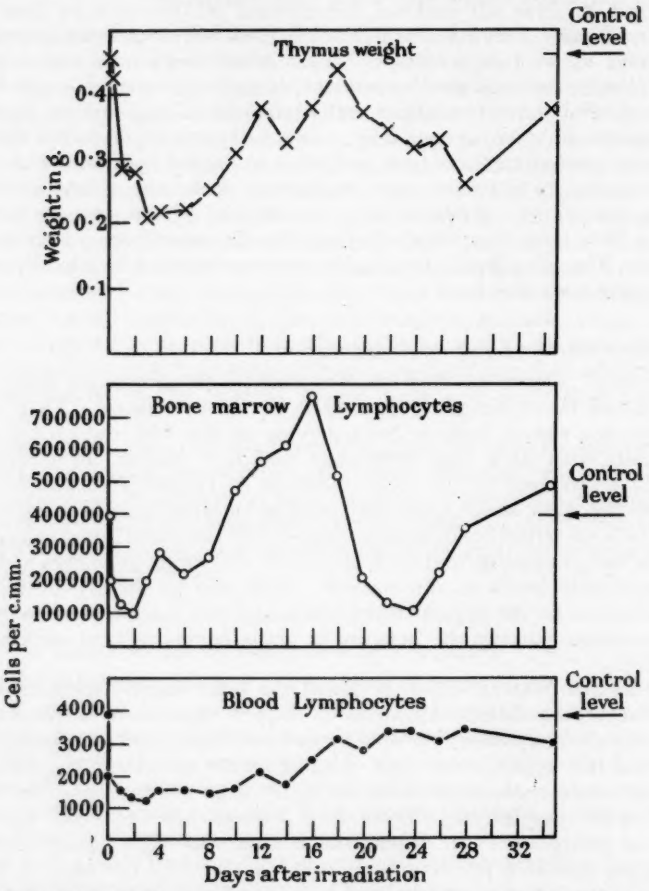


FIG. 2.—Changes in thymus weight, bone marrow lymphocytes and blood lymphocytes of guinea-pig after whole body irradiation.

the 4th hour to the 18th day it is $+0{\cdot}437$ ($p = < 0{\cdot}001$). From the 18th to the 28th day the correlation coefficient is $+0{\cdot}191$ which is not significant. The overall figure for the 118 observations appears to be weighted by those particularly between the 3rd and 18th days to produce a significant result.

The changes in thymic weight seem related, on histological evidence, to the lymphocyte content of the cortex. From the 4th hour to the 18th day a close

relationship exists between the lymphocyte level in the marrow and the lymphocyte content of the thymus. From the 18th to the 28th day the relationship between these two levels is not statistically significant.

These findings may be interpreted in several ways. Firstly, the bone marrow might be included as a lymphoid organ, forming substantial numbers of lymphocytes. These experiments show that its recovery pattern is similar to that of the thymus, namely, there is an "abortive" peak during recovery. Further, the recovery of lymphocytopoiesis in the marrow and thymus occurs simultaneously. Secondly, that the bone marrow is populated at a certain stage during recovery with lymphocytes formed in the thymus and other lymphoid tissues. These, reaching the marrow via the blood stream, make an essential contribution to its recovery, *e.g.*, function as stem cells (Yoffey, 1956). Thirdly, that the thymus and other lymphoid tissues are repopulated from the bone marrow. Fourthly, that the correlation is purely coincidental and the cells observed in the marrow during the recovery from irradiation are not, in fact, lymphocytes. These hypotheses will now be considered.

Production of lymphocytes in bone marrow.—Lymphocyte production is usually associated with diffuse or nodular lymphoid tissue which is not seen in guinea-pig marrow (Yoffey, 1954). However, if lymphocytes were formed in the marrow from a multipotent stem-cell they would be diluted amongst the many myeloid and erythroid cells, so explaining the absence of aggregations in the marrow. Certainly mitotic figures are rarely seen in marrow lymphocytes (Yoffey, 1954), since the vast majority are of the small type in which mitoses are not seen.

From 12 to 18 days after irradiation, assuming the bone marrow is a lymphoid organ, then lymphocyte production appears to be high. Thus lymphocytopoiesis seems to be firmly established in lymph nodes, at least normal in the thymus and above normal in the marrow. Although blood lymphocyte levels are increasing at this time, yet there is still a lymphopoenia. Brecher *et al.* commented upon an apparent anomaly in their experiments since a lymphopoenia persisted for a considerable time despite normal or even increased activity in lymphoid tissue between 7 and 14 days after irradiation. A similar observation is made by Jacobson (1954), who reviews the findings of several workers on the haematological effects of ionizing radiation. He believes such a discrepancy possible when the histological findings in the lymphoid tissue are purely qualitative.

In the present experiments both of the other large marrow groups, *i.e.* erythroid and myeloid, also showed peak levels in the marrow, well above normal, during recovery. This peak was rapidly reflected in the blood levels of these cells. In the erythroid group substantial numbers of immature cells appeared in the peripheral blood for several days and in the myeloid group a marked polymorphonuclear leucocytosis occurred. Likewise, if the marrow forms lymphocytes and discharges them at least normal blood levels, if not an elevation, might be expected, considering their peak level in the marrow and the activity in the other lymphoid tissues at this time. Thus blood lymphocyte levels during recovery from whole body irradiation are not necessarily a good index of the underlying state of activity in lymphoid organs. Not until after the 18th day, when thymic lymphocyte content and marrow lymphocyte levels were declining, did blood levels approach normal.

Sanders and Florey (1940) studied the effects of removal of lymphoid tissue in the rat and rabbit. Commenting upon the bone marrow picture in rats, after

removal of lymphoid tissue, in some there was evidence of lymphocytopoiesis but it was neither constant nor conspicuous. In only 1 rabbit were there nodules of lymphoid tissue and these were not large or numerous. Under such conditions definite evidence of compensatory lymphocyte formation in the marrow might be expected if it is an important source of lymphocytes.

Population of bone marrow by lymphocytes from lymphoid tissue.—Jordan (1939) suggested that lymphocytes are selectively filtered from the blood stream by the bone marrow and retained there. If, after whole body irradiation, they were withdrawn from the blood by the marrow at a faster rate than they were entering it, this may explain the lymphocytopoenia and gradual increase in lymphocytopoiesis in the thymus and lymph node until the lymphocyte requirements of the marrow have been met. Such a lymphopoenia following diversion of lymphocytes from blood into the marrow is analogous to the lymphopoenia which may follow diversion of lymphocytes from the blood by thoracic duct cannulation (Gowans, 1957).

A close morphological similarity was seen between lymphocytes in thymus and bone marrow smears from the present experiments. Cytoplasm tended to be minimal or absent, and when present was located at one pole of the cell. This form of lymphocyte probably represents a " resting " phase and differs from the living motile cell which, in culture, shows cytoplasmic evidence of amoeboid activity (Lewis and Webster, 1921). Further, there is a similarity in the nuclear chromatin of these cells which is less clumped compared with that of lymph-node lymphocytes. Downey (1948) noted a characteristic morphology of the thymus lymphocyte and describes the predominant thymic cell as being small with a very dark pachychromatic nucleus and absent or minimal cytoplasm.

EXPLANATION OF PLATES

FIG. 3.—Cell debris and pyknosis in cortex of guinea-pig thymus 4 hr. after whole body irradiation. (× 215.)

FIG. 4.—Depletion of cortex in guinea-pig thymus 24 hr. after whole body irradiation. (× 54.)

FIG. 5.—" Moth eaten " appearance of cortex in guinea-pig thymus, 4 hr. after whole body irradiation. (× 54.)

FIG. 6.—Small capillaries within cortex of guinea-pig thymus 12 days after whole body irradiation. (× 323.)

FIG. 7.—Capillary within cortex of guinea-pig thymus, containing a lymphocyte, 4 days after irradiation. (× 453.)

FIG. 8.—Cortex of guinea-pig thymus, 4 days after whole body irradiation. (× 11.)

FIG. 9.—Cortex of guinea-pig thymus, 16 days after whole body irradiation. (× 13.)

FIG. 10.—Cortex of guinea-pig thymus, 28 days after whole body irradiation. (× 13.)

FIG. 11.—Eosinophil granulocytes, including a mitotic figure, in the connective tissue of guinea-pig thymus 26 days after whole body irradiation. (× 550.)

FIG. 12.—Depletion of diffuse and nodular lymphoid tissue, in mesenteric lymph node of guinea-pig, 6 days after whole body irradiation. (× 6.)

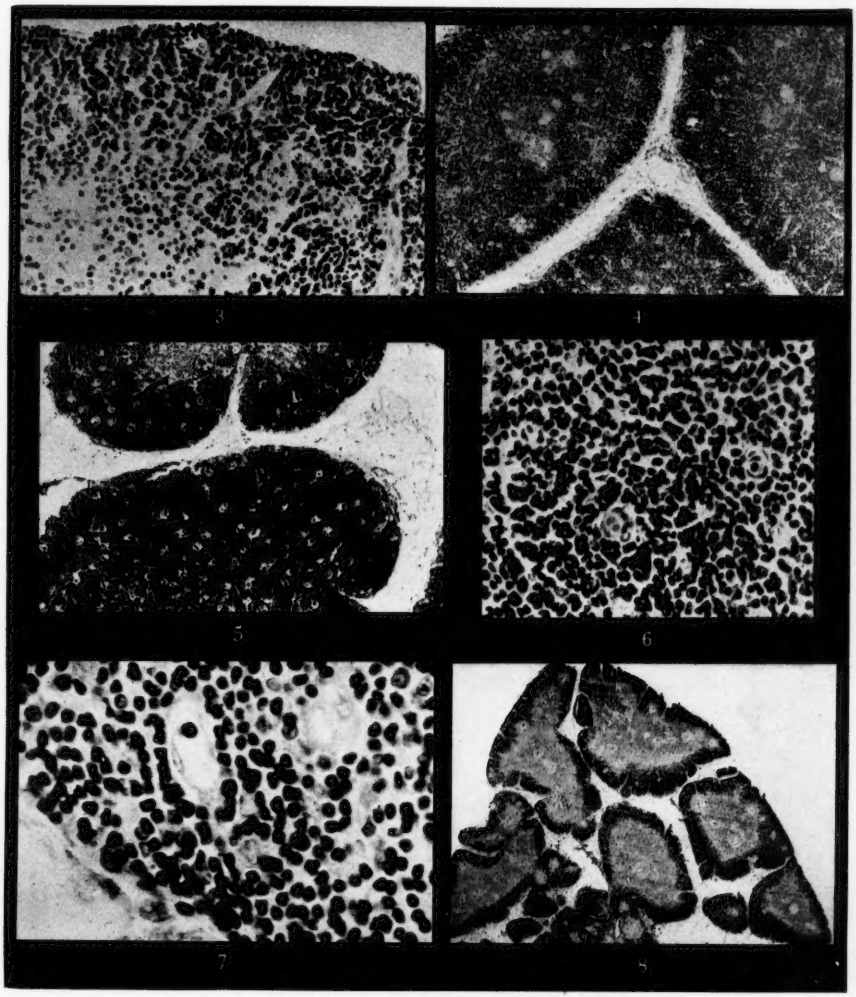
FIG. 13.—Re-formation of diffuse and nodular lymphoid tissue in mesenteric lymph node of guinea-pig, 10 days after whole body irradiation. (× 6.)

FIG. 14.—Diffuse and nodular lymphoid tissue in mesenteric lymph node of guinea-pig, 12 days after whole body irradiation. (× 6.)

FIG. 15.—Diffuse and nodular lymphoid tissue in mesenteric lymph node of guinea-pig, 18 days after whole body irradiation. (× 6.)

FIG. 16.—Section of bone marrow from guinea-pig, showing numerous small round cells, 14 days after whole body irradiation. (× 270.)

FIG. 17.—Section of bone marrow from guinea-pig showing numbers of small round cells, 16 days after whole body irradiation. (× 560.)



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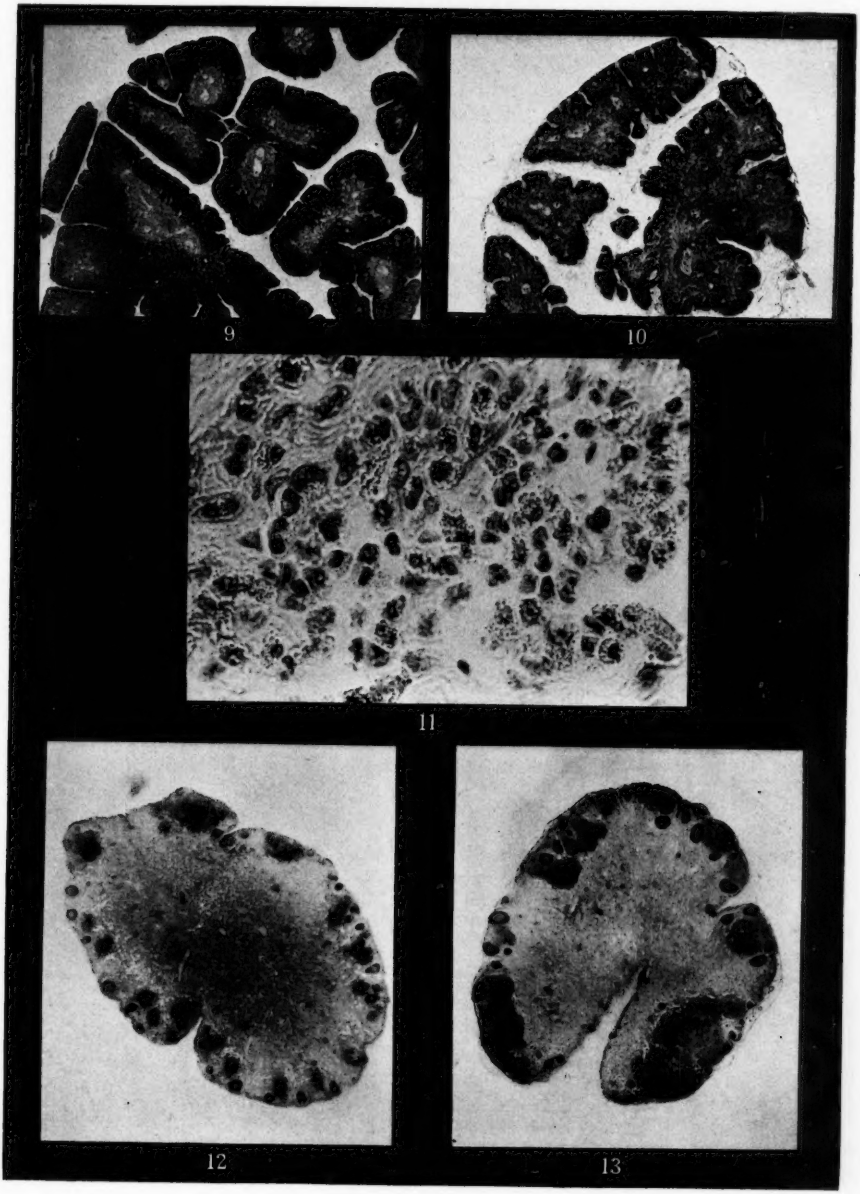

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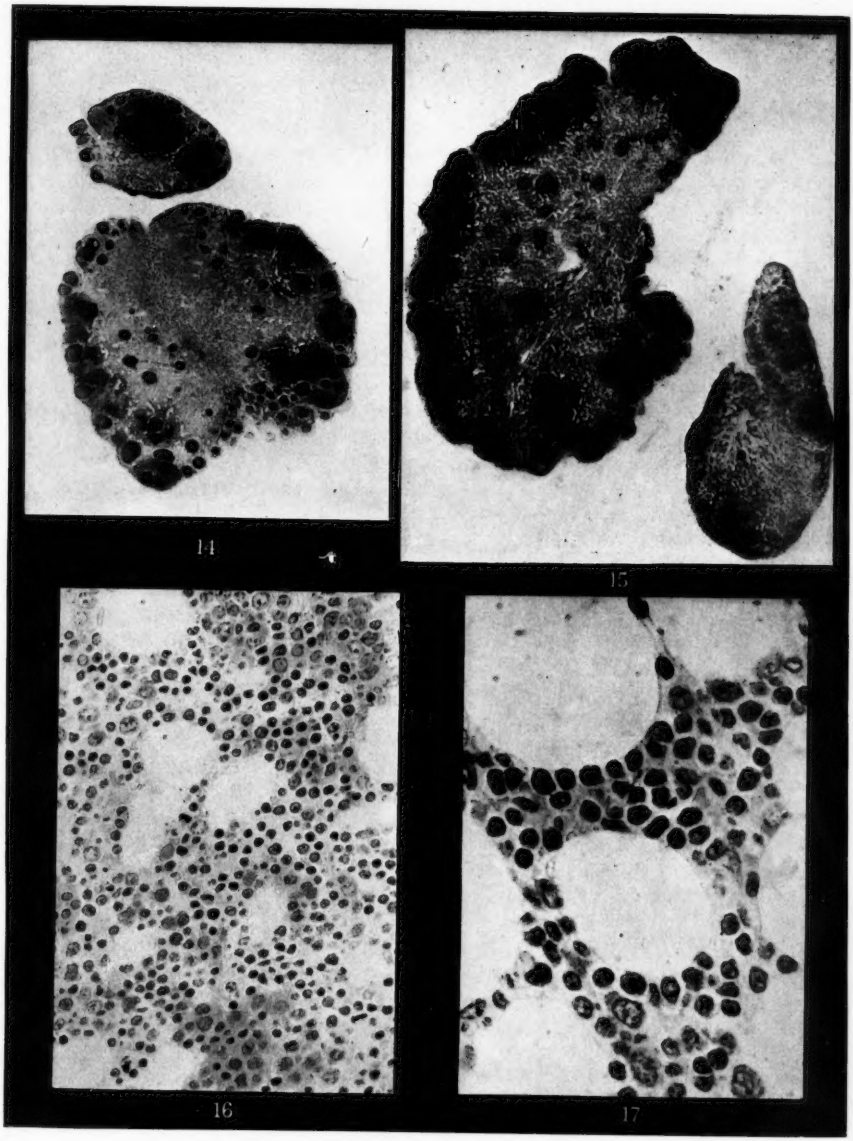

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Osogoe and Awaya (1956) followed the fate of lymphocytes transfused into rabbits whose bone marrow had been considerably depleted after saponin injection. A striking accumulation of lymphocytes was seen in the femoral and tibial marrow of each hind limb. They strongly deny any evidence of direct transformation of the accumulated lymphocytes into erythroid or myeloid cells.

The thymus may sometimes be the site of a lesion ultimately involving other haemopoietic organs. Simpson, Hempelmann and Fuller (1955) followed-up children given deep X-radiation for thymic enlargement during infancy. There was a higher incidence of malignant disease in treated children compared with a control group if untreated siblings. The neoplasias occurring most frequently were leukaemia (acute or subacute lymphocytic and acute stem cell) and thyroid tumours, especially carcinoma. McEndy, Boon and Furth (1944) investigated the role of the thymus, spleen and gonads in the development of leukaemia in a high-leukaemia stock of mice. The incidence of leukaemia in male and female mice was conspicuously reduced after thymectomy. Saxton, Boon and Furth (1944) investigated the effect of underfeeding in a high-leukaemia strain of mice. Leukaemia occurred at an earlier age and with greater frequency in the controls compared with those underfed. The decreased incidence in the underfed animals was thought to be partly due to hastened involution of the thymus.

To what extent could thymic lymphocyte production contribute to the lymphocyte content of bone marrow? Andreasen and Christensen (1949) concluded from mitotic counts in the rat over a wide age range that lymphocytopoiesis in the thymus is much greater than in other lymphoid organs. However, restoration of blood lymphocyte levels from the lymphopoenia resulting from starvation atrophy of lymphoid tissues was not accompanied by thymic regeneration. Restoration under these conditions, despite persisting thymic atrophy, was explained by compensatory activity in those lymphoid organs which had regenerated. Andreasen and Ottesen (1944), on the basis of nucleic acid turnover, believed the thymus to be by far the most important source of lymphocytes. From quantitative studies in young albino rats, Kindred (1942) estimated that the thymus produced almost 60 per cent of the number of lymphocytes produced by all the lymph nodes. Reinhardt and Yoffey (1956) investigated thoracic duct lymphocyte output in young male guinea-pigs after thymectomy. It may be concluded from their findings that the thymus is a major source of lymphocytes in the young guinea-pig.

Histological features of the thymus suggest lymphocyte production. Small lymphocytes are concentrated in the cortex of the lobules probably for two reasons. Firstly, there is a greater production of these cells in the cortex. Kindred (1942) noted a greater percentage of mitoses in the medium and large lymphocytes of the cortex than in those of the medulla. The cortex was where most of the mitotic figures were seen in material from the present experiments. Secondly, thymic lymphocytes may concentrate in the cortical region since accessible portals of exit, *i.e.* blood capillaries and lymphatics are here. Marine (1932) described the blood supply and lymphatic drainage of the thymus. Arteries are distributed to the cortex of the lobules, there forming a capillary network which finally converges upon medullary venous spaces. A lymphatic capillary network beneath the capsule of the lobule is formed from plexi in the lobule. Larger vessels arising from this network accompany blood vessels, especially those in interlobular septa. Kindred (1942) described a rich lymphatic supply of the rat thymus. Downey (1948) noted lymphatics, packed solidly with lymphocytes, adjacent to large blood

vessels in the thymus of a young rabbit 6 days after local thymic irradiation. Sections of guinea-pig thymus from the present experiments showed interlobular and intralobular lymphatics. Interlobular vessels were of two types : thin-walled and of considerable diameter or smaller vessels intimately related to the walls of arterioles—some being even within the tunica adventitia (cf. certain lymphatics of spleen described by Goldberg (1958)). The "moth-eaten" appearance sometimes seen in the cortex is partly due to the presence of thin-walled capillaries and is accentuated by the packing of small lymphocytes around them. Occasionally, besides red cells, they contain lymphocytes. Some of these lymphocytes were undoubtedly already in the blood entering the thymus, but bearing in mind the thin wall of the capillaries and motility of lymphocytes (Ebert, Sanders and Florey, 1940) they could directly enter these vessels by diapedesis. Sanders, Florey and Barnes (1940) concluded from experiments on the cat and rabbit that blood lymphocyte levels are not solely maintained by cells entering the blood via the thoracic duct.

Repopulation of the thymus and other lymphoid tissues from the bone marrow.—Loutit (1957 in considering the problem of radiation-leukaemia reviews work upon mouse lymphoma. He notes the well-established fact that donor myeloid cells can repopulate the haemopoietic tissues of a heavily irradiated recipient in whom the immunity mechanism has been depressed. Further, in the mouse, only those tissues containing myeloid cells, *e.g.*, bone marrow, spleen from young animals or embryonic liver are effective. There is little, if any, effect following injection of mouse thymus or lymph node. The injected material not only colonizes bone marrow and spleen but also the lymphatic tissues. Repopulation of the latter, Loutit suggests, may be interpreted in three ways. Firstly, that stem-cells from the marrow, "presumably reticulum cells", have greater potentialities than morphologically similar stem-cells in lymphatic tissue. Secondly, that certain myeloid tissue cells may de-differentiate and produce lymphatic tissue in place of their usual progeny. Thirdly, that a normally minor lymphatic component of myeloid tissue may be the source of regenerating lymphatic tissue. It is remarkable that only cell suspensions from myeloid tissue, and not lymphoid tissue, can repopulate the recipient's lymphoid tissue. Suspensions of lymphoid tissue must contain numbers of free cells capable of division. Such free cells, showing mitotic figures, were frequently seen in the intermediary sinuses of the medulla in guinea-pig lymph nodes recovering from irradiation. This rather negates Loutit's 3rd hypothesis and supports the first two, if it can be assumed that the "normally minor lymphatic component of myeloid tissue" is identical to lymphatic tissue elsewhere.

Loutit's interpretation of mouse lymphoma is that myeloid cells initiated towards malignant changes by irradiation, populate the thymus damaged by irradiation and in the presence of a neoplastic-promoting factor in the thymus undergo malignant change. Bearing in mind this hypothesis, the cases reviewed by Simpson *et al.* in which there was irradiation of the thymus must have received some irradiation of vertebral bone marrow.

Porter (1957) has shown in severely irradiated male rabbits repopulation of the marrow by female cells following injection of bone marrow from female donors. Restoration of blood lymphocytes and regeneration of lymphoid tissue was appreciably more rapid in these animals than in non-protected controls.

Congdon (1958) states that in mice given a lethal dose of irradiation followed

by injection of rat bone marrow, the thymus regenerates and is at first of the mouse type, later changing to the rat type. In these experiments upon guinea-pigs, the vast majority of lymphocytes accumulating in the marrow after irradiation were of the small type which do not show mitotic figures. There was no direct evidence to show that these cells were responsible for the repopulation of lymphoid tissue. Cells of lymphoblastic appearance were not seen in the blood of the irradiated animals, even in the period preceding and during recovery of lymphoid tissue. In only two cases were small numbers of atypical lymphocytes, resembling transitional cells of marrow, seen in the blood, at 14 and 16 days after irradiation, by which time recovery of lymphoid tissue was well established.

If lymphoid tissue is repopulated by myeloid elements then recovery of myeloid tissue should precede that of lymphoid tissue. Jacobson (1958) observes that following spleen shielding in totally irradiated animals, recovery of lymphatic tissue and bone marrow occurs at approximately the same speed. In these experiments upon guinea-pigs if the lymphocytes present in the marrow were formed there then recovery of lymphocytopoiesis in the marrow and thymus was concomitant. This favours their multifocal origin from a cell of equal radiosensitivity and radioresiliency, irrespective of site. The recovery of thymus and lymph node, together with the presence of large numbers of lymphocytes in the marrow preceded recovery of erythropoiesis and granulocytopoiesis. Warren *et al.* (1950) found recovery of the thymus preceded that of the marrow. From the 10th to the 15th day when thymic lymphocytopoiesis seemed normal, erythropoiesis and myelopoiesis in the marrow were only just commencing. Are lymphocytes functionally more significant than erythrocytes and granulocytes?

Coincidental correlation between two groups of non-related cells.—The small round cells accumulating in the marrow in large numbers during recovery from irradiation appear to be stem cells. Their presence in large numbers in the marrow preceded recovery of both the other main cell groups. A marked inverse relationship occurred between the neutrophil levels in the marrow and the small round cell levels. Clear transitions at close morphological intervals could be traced between them and typical blasts. These small cells may not in fact be lymphocytes but merely lymphocyte-like. Morphologically, they closely resemble the micromyeloblasts illustrated by Naegeli (1931) and Israels (1955). Evidence from these experiments that these cells are actually lymphocytes is twofold. Firstly, in smears and sections these small marrow cells cannot be morphologically distinguished from the small round cells of lymphoid tissue, especially the thymus. Secondly, there was evidence 4 hr. after irradiation that the radiosensitivity of the two cell groups was similar. Thus, if they are stem cells and not lymphocytes, then the radiosensitivity of these 2 cell groups is similar. Fruhman and Gordon (1955), injected adrenal steroids into rats and found a marked decrease in marrow lymphocytes. At the same time a marked thymolytic action was noted.

Significance of lymphoid tissue in recovery from whole body irradiation

How essential is lymphoid tissue to recovery from whole body irradiation? Kaplan and Brown (1957) in their experiments upon mice, noted that after the initial injury, when lymphoid tissue weights decreased, their animals could be divided into 2 groups. In one group, weights tended to swing upwards again and was an indication that the animal would survive and be capable of haemopoietic regeneration. In the other group, lymphoid tissue weights remained

even lower than those observed during the injury phase, although the standard errors were large on statistical analysis. Such animals appeared moribund at the time of sacrifice.

Porter (1957) noted that in rabbits dying in the first few days after large doses of whole body irradiation, the thymus had so shrunk that it was difficult to locate at autopsy and the spleen was also atrophic.

Trentin (1958) has noted that animals protected from lethal irradiation by injection of homologous or heterologous bone marrow, after apparent recovery from the immediate effects of irradiation tend to show late mortalities. Such deaths are preceded by progressive loss of weight and fibrosis of lymphoid organs which had initially recovered and even shown temporary hyperplasia.

Examination of marrow smears from the small number of guinea-pigs dying after the dose of irradiation used in these experiments revealed a markedly hypocellular marrow. The few cells present were mostly mature granulocytes and monocytes, with occasional lymphocytes. The presence of large numbers of lymphocytes in the marrow of guinea-pigs after whole body irradiation seemed to presage ultimate haemopoietic recovery.

SUMMARY

Young male guinea-pigs were given a large dose of whole body gamma irradiation and quantitative cellular examinations made on the bone marrow at close intervals, from 4 hr. after irradiation until haemopoiesis in the bone marrow had returned to normal. At the same time, thymic weight was recorded and also sections of marrow, thymus and mesenteric lymph node examined histologically.

Statistically significant weight changes which, on histological evidence, appeared to be due to variations in cortical lymphocyte content, were noted in the thymus during recovery from irradiation. Statistically significant changes were detected in bone marrow lymphocyte levels during the recovery period. The manner of recovery in the thymus and bone marrow was similar, both showing an abortive increase in lymphocyte levels. This was followed by a decline in their lymphocyte content with ultimate return to normal levels by 35 days after irradiation. This recovery pattern was not detected in the mesenteric lymph node. The significance of the similarity in recovery of thymus and marrow lymphocyte levels is discussed.

Recovery of lymphocytopoiesis in the thymus and mesenteric lymph node, together with restoration of marrow lymphocyte levels preceded recovery of myeloid elements in the bone marrow. The blood lymphocyte levels during recovery from whole body irradiation were not good indices of the underlying activity in the lymphoid tissues examined, particularly the thymus.

There was evidence of granulocytopoiesis in thymus sections from animals in the later stages of recovery.

I wish to thank Professor J. M. Yoffey for helpful criticism during the preparation of this paper and for research facilities : also Dr. R. C. Tudway for permission to use equipment in the Radiotherapy Department, Bristol General Hospital and the hospital physicists for their constant assistance. I am grateful to Miss J. A. Drury for technical assistance and to Mr. M. Maggs for the photographs.

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ACQUIRED TOLERANCE TO HOMOLOGOUS TRANSPLANTATION OF ENDOCRINE GLANDS IN INBRED STRAINS OF MICE*

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ACQUIRED immunological tolerance, having been demonstrated to occur in several species of animals, now seems to be a well established biological phenomenon (Billingham, Brent and Medawar, 1953, 1956; Woodruff and Simpson, 1955; Hasek and Hraba, 1955). As usually carried out, tolerance is induced by injecting the prospective recipient with viable cells from the prospective donor by the intraperitoneal or subcutaneous route during embryonic life or by the intravenous route shortly after birth. Normal tissues successfully transplanted following prior treatment to produce tolerance in the prospective hosts have been skin in mice (Billingham *et al.*, 1953, 1956), skin in rats (Woodruff and Simpson 1955; Egdahl, Good and Varco, 1957), skin in fowl (Hasek, 1956), skin in dogs (Hasek, 1957), ovaries in mice (Martinez, 1956), and adrenals in mice (Medawar, 1958). In addition, mice have been made tolerant to transplantable adenocarcinoma by the neonatal injection of the tumour itself or by injection of viable spleen cells from the prospective donor strain prior to delivery.

With the successful homotransplantation of organs as indicated by morphological survival in the tolerant recipient it becomes important to evaluate their physiological function when they are residing in a genetically foreign host. Preliminary observations from our laboratories (Martinez, 1956) indicated that castrated female mice of the ZCe F_1 stock, tolerant of A tissue induced by intraperitoneal injection of A spleen cells in last gestation, accepted ovaries derived from A strain donors. Further, study of the cyclic changes in vaginal epithelium indicated that the transplanted ovaries expressed functional activity for a minimum of 45 days.

Following transplantation of adrenal glands in tolerant adrenalectomized mice, evidence of persistent function of the transplanted glands has been obtained (Hasek, 1957). To date no studies on homotransplanted pituitary glands have been made.

It seemed of importance to determine whether the hypophysis could be successfully homotransplanted into immunologically tolerant hypophysectomized recipients and to evaluate the functional integrity of both hypophyseal and ovarian homotransplants. Further, in view of recent findings revealing differences among mice strains both in ability of their cells to evoke a tolerant state and

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differences among recipient strains in capacity to develop tolerance (Billingham and Brent, 1957 ; Martinez, Smith and Good, 1958), we have extended our studies to several strains of inbred mice and different donor-host combinations.

It is the purpose of this report to present experiments defining the successful transplantation of both ovaries and hypophyses when immunological tolerance has been achieved by the intravenous injection of spleen cells immediately after birth, and to present evidence of active physiological function of the transplanted endocrine organs during prolonged periods of residence in the tolerant recipient of different genetic make-up.

MATERIALS AND METHODS

Mice of the Z (C3H), Ce, C57 Bl and BALB/C inbred strains and ZBC and ZCe F_1 hybrids were used. Acquired tolerance was induced by the method described by Billingham and Brent (1957) consisting essentially in the intravenous administration into newborn mice of a given strain (no later than 24 hr. after birth) of viable spleen cells taken from adult donors of a homologous strain. The exact method of preparation of the cell suspension, number of cells injected and the technique of injection have been described elsewhere (Billingham *et al.*, 1956 ; Martinez *et al.*, 1958). Animals surviving the intravenous injection were raised by their own mothers and weaned at 25 to 30 days of age. After weaning, mice were kept on our standard laboratory fox chow diet and tap water.

Acquired tolerance to homologous transplantation of ovaries

In these experiments the following donor-host strain combinations were used : BALB/C → ZCe F_1 ; Ce → ZBC ; Ce → Z(C3H) and Z(C3H) → Ce. At approximately 60 days of age the injected mice were submitted to bilateral ovariectomy through a lumbar approach followed immediately by the transplantation of 2 ovaries taken from female mice of the strain corresponding to that from which the spleen cells were derived. Donors were of the same age as the recipients.

Ovarian transplantation was performed in the following way ; upon sacrificing the donor, both ovaries were removed and placed temporarily in a Petri dish containing Ringer-Locke's solution. In this medium the glands were freed of their capsules under a dissecting microscope and transplanted to the axillary region of the recipient animal. The grafts were placed under the right pectoral muscle through a small incision subsequently closed with a single silk suture.

Starting at 10 to 15 days following surgery, samples of the vaginal content were taken from each animal to determine the presence or absence of oestrous cycles. This was done at intervals of 10 days and samples were made daily for 4 consecutive days. The method consisted of injecting a drop or two of physiological solution of sodium chloride into the vagina, aspirating the cell suspension obtained, smearing on a clean glass slide and examining this preparation under the microscope after staining with 1 per cent methylene blue.

The stages of the oestrous cycle reflected by changes in vaginal cells were diagnosed under the following categories :

0 = anoestrous indicated when all the cells on the vaginal smear are leucocytes.

1 = transition to proestrous, when almost all the cells on the vaginal smear are leucocytes, but a few nucleated epithelial cells are observed on the smear.

2 = proestrous when leucocytes occurred together with many nucleated epithelial cells on the vaginal smear.

3 = suboestrous when nucleated epithelial cells, leucocytes and non-nucleated squamous cells (scales) comprise the cell population on the vaginal smear.

4 = oestrous—when non-nucleated squamous cells or scales were the only cells present on the smear.

5 = metaoestrous, when scales and leucocytes occur together on the smear and scales are present in groups with shingle-like arrangement.

6 = transitional stage when a few scales and mostly leucocyte are present on the smear.

Such preparations were found to be reliable in diagnosing the particular stage of the oestrous cycle in control mice, and it was presumed that the characteristic cellular morphology of the vaginal smears reflected the same basic functional activity of the ovaries in the animals bearing ovarian homotransplants. Two to 3 months following ovarian transplantation the grafts were removed surgically for histological study and further daily observations of the vaginal changes were continued throughout a period of 15 days.

Ovariectomized mice not treated at birth with homologous spleen cells were also transplanted with homologous ovaries and served as controls.

Acquired tolerance to homotransplantation of hypophyseal gland

In this experiment female mice injected at birth with homologous spleen cells were used. The donor-host strain combinations were as follows: CBA → $ZCeF_1$; C57 Bl → ZCe F_1; BALB/CA F_1 and Ce → ZBC. Approximately 2 months following the injection of spleen cells, recipient animals were submitted to total hypophysectomy by the technique described by Thomas (1938) and slightly modified by Ferguson (personal communication). This was followed at the same sitting by the transfer of the pituitary graft into the emptied sella turcica as was previously described by Martinez and Bittner (1956). The donors of the graft were mice of the same strain as those used for spleen injections and of the same sex and age as that of the host.

The success of the hypophyseal transplantation was judged by the ability of the host to maintain growth and increase its body weight and the presence of a normal sexual function as indicated by the study of the vaginal oestrous cycles. In addition, in an isolated instance a hypophysectomized animal made tolerant by injection of homologous cells at birth and transplanted with homologous hypophysis into the sella turcica from a mouse of the donor strain, the ability to conceive, maintain pregnancy, deliver normal offspring and lactate was studied.

After surgery mice bearing pituitary homografts were housed individually and weighed at weekly intervals for a period of 2 months. Vaginal smears were taken from each mouse at intervals of 2 weeks at which time smears were made on 4 consecutive days to determine the presence or absence of oestrous cycles.

Groups of ZBC and ZCe F_1 female mice not injected at birth with homologous spleen cells received intracellar hypophyseal homografts taken from Ce and BALB/C donors respectively and served as controls. Study of growth and of oestrous activity were performed in the same way as in the experimental groups.

RESULTS

Tolerance to ovaries

The results of this experiment are summarized in Table I. Of 4 mice of the ZCe F_1 strain treated at birth with BALB/C spleen cells, 2 accepted subsequent homotransplantation of BALB/C ovaries. These 2 animals exhibited normal and complete oestrous cycles for 2 months as long as the grafts were kept in place.

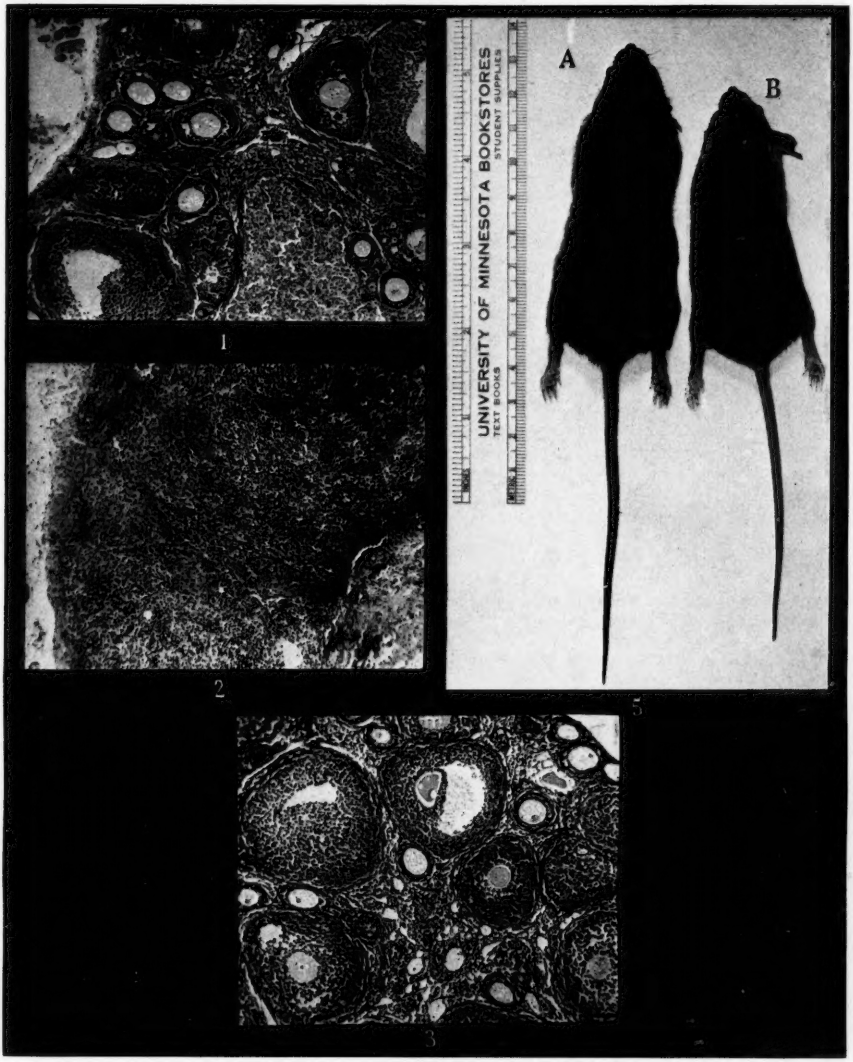
EXPLANATION OF PLATE

FIG. 1.—Ce ovary trasplanted into tolerant ZBC recipient. The ZBC mouse had been injected intravenously at birth with viable Ce spleen cells obtained from an adult donor. Note integrity of histology of the ovary and absence of inflammation one month after transplantation. × 66.

FIG. 2.—Ce ovary into non-tolerant ZBC recipient. The recipient animal was not injected at birth. Note distorted architecture of the ovary and inflammatory infiltration of the gland. × 66.

FIG. 3.—Normal control Ce ovary taken from an adult Ce female mouse. × 66.

FIG. 5.—Difference in size between a hypophysectomized ZBC mouse tolerant to Ce, successfully homotransplanted with Ce pituitary (A) and a hypophysectomized non-tolerant mouse of the same strain in which a hypophyseal homograft failed to take (B). The picture was taken 4 months following transplantation of the pituitary.



Martinez, Smith and Good.

Upon the removal of the graft the evidence of normal sexual activity ceased and the vaginal epithelium revealed persistent anoestrous. Of 19 mice of the ZBC stock pretreated with Ce spleen cells, all accepted the Ce ovaries as indicated by the presence of oestrous cycles, during the entire period the grafts were left in place (2–3 months). Here again the removal of the transplanted ovaries resulted in a cessation of vaginal activity in every instance. Similar results were obtained in 5 mice of the Z(C3H) strain pretreated with Ce spleen and in 6 animals of the Ce strain injected at birth with Z(C3H) strain cells.

TABLE I.—*Ovarian Homotransplantation in Tolerant Mice*

Donor strain	Host strain*	Number of mice with ovarian homografts	Number of mice showing persisting oestrus cycles	Per cent
Tolerant mice				
BALB/C	ZCe F_1	4	2	50
Ce	ZBC	19	19	100
Ce	Z(C3H)	5	5	100
Z(C3H)	Ce	6	6	100
Total	—	34	32	94
Controls†				
Ce	ZBC	12	0	0
BALB/C	ZCe F_1	14	0	0
Total	—	26	0	0

* Female mice pretreated with homologous spleen cells intravenously at birth, castrated at 2 months of age and transplanted with ovaries from the donor strain originally providing the spleen cells.

† Female mice castrated at 2 months of age and transplanted with ovaries from the donor strain indicated.

As it would be expected, all 12 castrated ZBC control females failed to accept Ce ovaries as determined by the absence of vaginal changes reflecting ovarian function. In these animals the vaginal smears always revealed anoestrous. Similarly, uniform failures of the ovarian homotransplantation occurred in 14 control mice of the ZCe F_1 strain transplanted with ovaries of the BALB/C strain.

Figs. 1, 2 and 3 illustrate the microscopic appearance of ovaries in homotransplants of tolerant and non-tolerant recipients. These ovaries were removed one month following homotransplantation for comparison with an ovary from a normal mouse of the same strain and age. Fig. 1 shows the Ce strain ovary one month following homotransplantation into the castrated ZBC host which was previously made tolerant by the intravenous injection of Ce spleen cells at birth. Fig. 2 shows the transplanted Ce ovary from a non-tolerant castrated ZBC recipient. Fig. 3 shows the microscopic characteristics of an ovary from a normal Ce mouse 3 months of age. The normal histological appearance of the Ce ovary surviving one month in the tolerant ZBC recipient contrasts sharply with the Ce ovary in the non-tolerant recipient, which reveals only scar tissue and evidence of inflammatory reaction. Numerous follicles in all stages of development are to be seen in the ovary of the tolerant recipient, while no evidence of follicle formation is to be seen in the Ce ovary in the non-tolerant ZBC recipient.

Tolerance to hypophysis

The results of this experiment are summarized in Table II. All three mice of the ZCe F_1 stock treated at birth with CBA spleen cells accepted hypophyseal homografts following hypophysectomy, as judged by the subsequent progressive increase in body weight and the presence of normal oestrous cycles observed in these animals. On the other hand, of the group of ZCe F_1 mice pretreated at birth with C57 Bl spleen, one out of three accepted C57 Bl pituitaries and of 2 mice of the same strain (ZCe F_1) treated with BALB/CAF_1 spleen at birth, only one accepted subsequent hypophyseal grafts from BALB/CAF_1 donors. Finally, of a group of 12 ZBC mice receiving Ce spleen cells at birth, 10 became tolerant and accepted homologous pituitary grafts from donors of the Ce strain.

TABLE II.—*Hypophyseal Homografts in Tolerant and Non-Tolerant Mice*

Donor strain*	Host strain	Number of mice grafted	Number accepting the graft†	Per cent
CBA	ZCe F_1	3	3	100
C57 Bl	ZCe F_1	3	1	33
BALB/CA F_1	ZCe F_1	2	1	50
Ce	ZBC	12	10	83
Total	—	20	15	75
	Controls			
Ce‡	ZBC	6	0	0
CBA	ZCe F_1	10	0	0
Total	—	16	0	0

* As judged by a progressive increase in body weight and cyclic vaginal activity.

† Spleen cells from this strain injected at birth, and subsequently (2 months) hypophysis taken from animals of same strain for transplantation into recipient of spleen cells.

‡ Hypophysis used for homotransplantation.

As is seen in the table none of the control mice accepted homologous transplants of hypophyseal glands. This fact was indicated by their failure to grow, by the continuous loss in body weight and absence of evidence of oestrous cycles on study of the vaginal smears from these animals following hypophysectomy and homotransplantion of hypophyseal gland. Fig. 4 compares the growth of hypophysectomized tolerant mice successfully homotransplanted with pituitary gland and the failure of growth and the weight loss which regularly occurred in the non-tolerant control mice which were hypophysectomized and transplanted with homologous pituitary glands. This observation is further illustrated in Fig. 5 which compares the size of a hypophysectomized tolerant mouse in which the pituitary gland was successfully homotransplanted and a hypophysectomized non-tolerant animal in whom the homotransplantation of hypophysis failed.

Finally, one hypophysectomized ZCeF_1 female originally made tolerant by injection of CBA spleen cells at birth and subsequently transplanted with CBA hypophysis showed normal growth, normal oestrous cycles, and has been successfully bred to a normal ZCeF_1 mouse. This animal conceived, had a perfectly normal pregnancy, and has delivered normal offspring. In addition, the offspring was nursed by the mother which suggests that successful lactation occurred.

DISCUSSION

The results obtained in this study are of interest from several points of view. In the first place they demonstrate that functionally active ovaries and hypophyses can result from homotransplantation of these glands into immunologically tolerant recipients. In addition, they demonstrate that these transplanted endocrine glands maintain an essentially normal histology whereas glands transplanted under similar circumstances to non-tolerant recipients do not function and are destroyed with accompanying inflammation and fibrosis. According to our

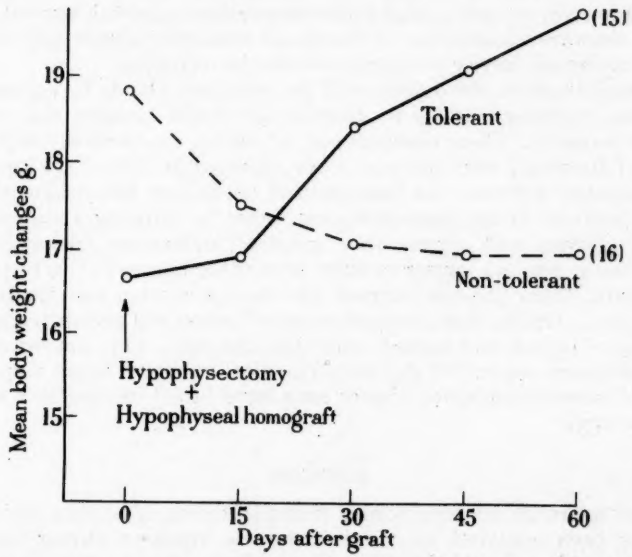


FIG. 4.—Growth of tolerant and non-tolerant hypophysectomized mice receiving pituitary homotransplants. (Between parentheses number of animals.)

findings, homotransplanted ovaries and hypophyses have retained their respective functions as long as they were permitted to reside in the foreign host. In these experiments the periods of function have been up to 3 months for ovaries and from 2 to 4 months for the hypophysis. In the case of the ovarian homotransplants, success was obtained in all donor-host strain combinations tried. Success of the ovarian homotransplantation was demonstrated both by the maintenance of cyclic oestrous activity and by the histological studies demonstrating normal architecture of the gland and normal follicle formation in homotransplants in the tolerant recipient. However, here as in skin homotransplantation studies, a difference in host-donor strain combinations is suggested. Whereas 100 per cent successful transplantation was observed in the strain combinations Ce → Z(C3H) and Z(C3H) → Ce, only half of the ZCe F_1 hybrids treated with BALB/C spleen cells were subsequently tolerant of BALB/C ovaries. In spite of the small number of animals in this series, it is of interest that these results are consonant with the

previously reported refractoriness of ZCe F_1 mice to the induction of immunological tolerance with BALB/C spleen cells when skin homotransplantation was used as the indicator system (Martinez, *et al.* 1958).

Pituitary homotransplantation in the immunologically tolerant host revealed successful transplantation in hypophysectomized animals in a frequency comparable to that previously reported for pituitary isografts (Martinez and Bittner, 1956). The indications of successful pituitary transplantation were the re-establishment of normal growth and weight gain, the presence of normal ovarian function and in one host, normal conception pregnancy, birth process and lactation. This last observation has been made in only one animal, and studies to confirm it are in progress. However, we believe that these observations establish beyond reasonable doubt that homotransplantation of functional endocrine organs may be achieved if the immunological barrier to transplantation is overcome.

In contradistinction, the ovaries and the pituitary glands transplanted across genetic strain barriers in mice to animals not made tolerant did not survive or function normally. These observations, of course, run contrary to the original postulates of Halsted (1909) but were to be expected in light of the known lack of histocompatibility between the transplanted tissue and the recipient host, and the known activity of the immunological forces in bringing about rejection of foreign cells, tissues and organs when genetic barriers are crossed in grafting. Endocrine tissue does not appear to differ from other tissues in this respect.

These data, then, provide support for the speculation previously made by Billingham *et al.*, (1956), that immunological tolerance will permit transplantation and function of organs and tissues other than the skin. This conclusion provides hope that ultimate control of the immunological rejection forces responsible for sloughing of homotransplanted tissues may have broad implications in the field of endocrinology.

SUMMARY

Acquired tolerance for homologous transplantation of ovaries and pituitaries in mice has been achieved by the intravenous injection during the neonatal period (within 24 hr. after birth) of homologous living spleen cells taken from adult mice of the same strain as the prospective donors of the endocrine glands.

It was found that in the immunologically tolerant host the homotransplanted glands retained their functional endocrine activity as well as anatomical integrity. In case of ovaries the functional adequacy was reflected in the occurrence of normal cyclic changes in vaginal cells. In case of the pituitaries the maintenance of normal growth, the presence of ovarian function as reflected in cyclic changes in vaginal cells and in one instance the occurrence of conception, gestation and lactation were the criteria reflecting the physiological function of this organ.

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TUBERCULIN THERAPY OF MOUSE THIGH TUBERCULOSIS

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THROUGH its intensity and duration, the quarrel over the therapeutic value of tuberculin in tuberculosis has become a byword of medical history. The wide differences in the results obtained and the views expressed regarding the treatment of human disease have been summarised at different times in their books by Hamman and Wolman (1912), and Rich (1951). Many animal studies, mostly in the guinea-pig, and directed primarily at elucidation of the relationship between hypersensitivity and immunity, have been conducted. A number of these have been reviewed by Willis, Woodruff, Kelly and Voldrich (1938) and Rich (1951). Despite the vast amount of labour devoted to this work, most of the difficulties and differences of opinion remain, and it appeared that some clarification might emerge from observations on the response to tuberculin treatment of a lesion of which the whole course could be followed in detail.

Selbie and O'Grady (1954) showed that the intra-muscular inoculation of *Myco. tuberculosis* into the mouse thigh resulted in a local tuberculous lesion, the course of which could be followed by measurement. Similar infection of the other thigh in animals suffering from this lesion produced a swelling which at 24–48 hr. was very much larger than that resulting from the same inoculum in control animals. Coincident with this hypersensitive response in the freshly infected thigh, a transient fall occurred in the size of the established lesion in the other leg.

A similar fall in size of the lesion was also produced by the injection of tuberculin into the other thigh and it appeared that by repeated inoculation of tuberculin at suitable intervals, lasting and possibly permanent reduction in size of the tuberculous lesion might be obtained. In these animals, the repeated injection of tuberculin gave rise to a complex series of changes which have already been described (O'Grady, 1957). The extent to which control of the lesions was obtained by this treatment is now reported.

METHODS AND MATERIALS

Inoculum.—The animals were infected with the $H_{37}Rv$ strain of *Mycobacterium tuberculosis* grown for 21 days on Youman's modification of Proskauer and Beck's medium. The surface growth was harvested and a stable suspension prepared in Dubos' medium without the addition of albumin, by a combination of Griffith's tube homogenisation and mechanical shaking. This suspension was standardised by opacity to contain about 10^9 organisms per ml.

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Infection.—Using a 1-ml. syringe and intradermal (gauge 26) needle, groups of 10 Strong A albino mice were inoculated in the left thigh with 0·1 ml. of this suspension, or of the suspension diluted 1/100 with Dubos' medium without albumin. The animals, distinguished by dye marks, were housed in groups of 10. They were fed once daily on mouse cubes, and water was supplied as required.

Tuberculin treatment.—Groups of animals at both dose levels began treatment with tuberculin 10 days and 27 days after infection. Other groups of animals infected with undiluted inoculum began treatment 35 days after infection. For controls, each treated group was paired with a similar group of litter mates infected at the same time with the same inoculum. Intramuscular injections of old tuberculin, diluted 1/5 with Sorensen's buffer pH 7·4, were given into the right thigh at alternating 3 and 4 day intervals (Mondays and Thursdays). Each group received 10 or 11 injections of tuberculin, the whole period of treatment lasting between 34 and 39 days. The experiments were repeated 3 times for each period of tuberculin treatment at both dose levels.

Measurement.—The course of the lesions was followed as previously described (Selbie and O'Grady, 1954) by measuring the greatest medio-lateral width of the thigh with sliding calipers. In some of the experiments, the infected thighs were measured immediately after injection, at 15 and 30 min., and at 1, 2, 5, 12, and 20 hr. All the infected thighs were measured daily and the average size of the lesions in each group calculated. The average size of the lesions in the treated groups was then expressed as a percentage of the average size of the lesions in the corresponding controls.

RESULTS

The course of untreated lesions of this type has been previously described (Selbie and O'Grady, 1954). This course was followed closely by the lesions in the control groups in the present experiments. After infection with the undiluted inoculum there was a period of establishment, lasting 10 or 15 days, during which time very little change in the size of the infected thigh occurred. This was followed by a period of progression during which the lesions actively increased in size for a further 30 to 50 days. Following this, the lesions entered a period of spontaneous resolution, often associated with the development of sinuses, and the thighs approximated to normal size again after a further 70 or 80 days. The times at which tuberculin treatment, was instituted (10, 27 and 35 days) corresponded, therefore, with the end of the period of establishment, and with early and late stages in the period of progression. The inoculum diluted 1 : 100 was the smallest dose which gave rise to measurable lesions in the animals. These small lesions reached their maximum size earlier than those in the heavily infected animals. For this reason, no groups infected with this inoculum began treatment with tuberculin at 35 days since at this time resolution had already begun.

Injection of tuberculin into the thighs of these animals usually gave rise to a reaction having two components, one maximal at about 15 min. and the other appearing after about 6 hr. and reaching its maximum in about 24 hr. In the animals infected with the undiluted inoculum, the first two reactions to tuberculin showed no delayed component and it was not until a third injection was given that a reaction showing both components was obtained. The subsequent changes in reaction to repeated tuberculin have been previously described (O'Grady, 1957).

The transient response

No reproducible change occurred in the size of the lesions during the rapid component of the response to tuberculin. In all groups with progressive lesions, whether a delayed tuberculin reaction was obtained or not, a transient fall in the size of the lesions occurred which frequently lasted for some days (Fig. 1).

Repeated inoculation

The summation of reactions of this kind led to very variable results and no clear pattern of relationship in detail between succeeding reactions could be seen (Fig. 1). Nevertheless, the overall changes in size of the lesions followed a definite course.

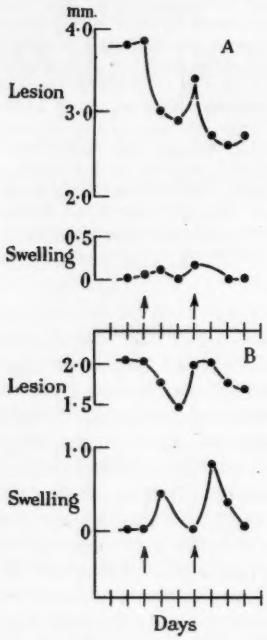

FIG. 1.—Examples of the fall in size of lesions in response to the first two injections of tuberculin. A: Unaccompanied by local tuberculin reaction. B: Accompanied by local tuberculin reaction (minimally infected animals).

Overall Response in Heavily Infected Animals

Treatment in stage of establishment.—The lesions rapidly fell in size to reach 50 per cent of the control value within a week. After this time, very little further change in size occurred. Cessation of treatment was followed by a rapid return to the control size (Fig. 2A).

Treatment in stage of progression.—A fall to about 40 or 50 per cent of the control size usually occurred during the first 10 days of treatment after which there was a gradual increase in size of the lesions in spite of the continuing therapy. In some groups, the lesions ultimately became larger than those in the control animals. In animals in which treatment was instituted later in the stages of progression, a fall in size followed by a rise was again seen but in no group did the lesions become larger than those of the controls (Fig. 2B and C).

Overall Response in Minimally Infected Animals

Treatment in stage of establishment.—Extremely variable responses were obtained in these small lesions from group to group. In one group of animals the lesions were at one period almost abolished but in others no detectable change occurred. In the group showing extreme reduction in size, " escape " from the tuberculin therapy was again seen (Fig. 3A).

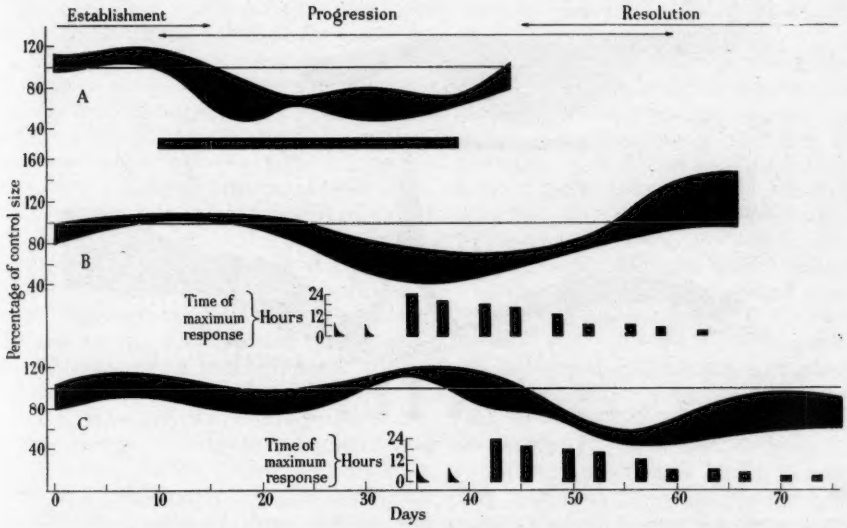

FIG. 2.—Response of lesions in heavily infected animals to repeated tuberculin beginning: A, at end of stage of establishment, and B and C, in stage of progression. In A, the black line shows the period of treatment. In B and C, the individual injections are indicated and the time at which the tuberculin response was maximal is shown. In these animals the first two injections of tuberculin did not give rise to reactions with delayed components.

Treatment in the stage of progression.—These animals gave one of the most interesting results. Again, in actual magnitude the results were very variable, but a pattern of response was seen in these animals which was not otherwise encountered. In almost all the animals, the first inoculation of tuberculin produced an increase in the size of the lesions and this response was seen to an extreme degree in one group of animals in which the lesions rose to almost three times the size of the controls and then subsided within 4 days. Animals showing this reaction invariably fared worse than their controls. The lesions in the group in which this reaction was most developed were twice the size of the controls at the end of the period of tuberculin treatment (Fig. 3B). One group (hatched in the figure) failed to show this response and in this group, a gradual fall in size followed by rapid " escape " from the tuberculin therapy occurred.

DISCUSSION

The complex responses of these animals may help to explain some of the wide differences of opinion which have been held regarding the efficacy of tuberculin therapy. Even amongst those who have believed tuberculin treatment to be of value, there has been considerable disagreement about the mechanism of its effects and therefore about the way in which the treatment should be applied. It has been known from the earliest days that the administration of tuberculin to

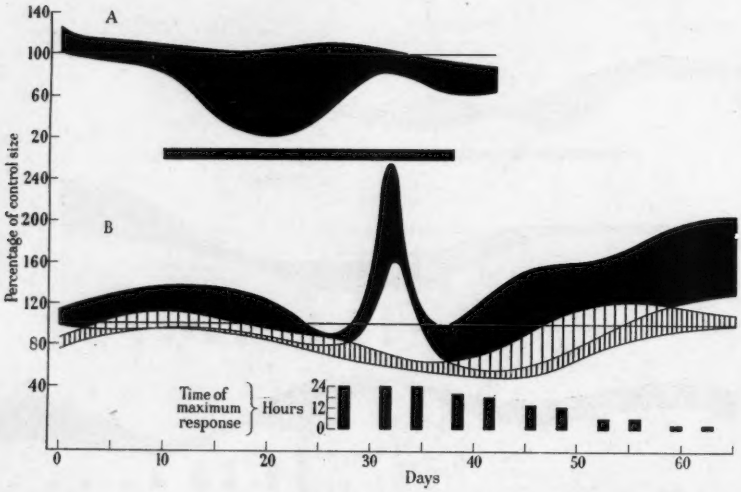

FIG. 3.—Response of lesions in minimally infected animals to repeated tuberculin beginning: A, early and B, late in the progressive stage. In A, period of treatment is shown by the black line. In B, the individual injections are indicated and the times at which the tuberculin response was maximal is shown. The hatched area shows the course of the lesions in a group of animals in which focal reactions did not occur.

a tuberculous animal can give rise to local, focal and general reactions. A number of exponents of tuberculin therapy (Riviere and Morland, 1913; Bandelier and Roepke, 1913) regarded the focal reaction around existing tuberculous lesions, with its accompanying changes in blood supply and cellular infiltration as the essential, beneficial object of tuberculin therapy. Since Virchow's (1891) observations, a great deal of evidence has been gathered to show that such focal reactions in fact commonly lead to spread of the disease.

Rich (1951) has summarised the evidence in favour of the view that the object of tuberculin therapy is the abolition of hypersensitivity. "Improvement", he says, (p. 559) "parallels de-sensitization". Other workers, including some recent ones (Smith and Vollum, 1956; Howells and Swithinbank, 1957) have focused attention on the importance of the reaction to tuberculin itself in bringing about improvement. The principal evidence that the reaction itself and not the development of desensitisation is required is that improvement may follow the very earliest injections of tuberculin (Krasso, 1935; Smith and Vollum,

1950) and that the beneficial result may decline with declining reactions as desensitization develops (Howells and Swithinbank, 1957).

The possible contribution of the mouse thigh lesion to this topic is best seen by distinguishing three types of responses. Firstly, a fall in size of the existing lesion occurred in response to the *first* injection of tuberculin. Secondly, with repetition of tuberculin, this fall became progressively less until further tuberculin had no effect, and the lesions increased again to the control size. Thirdly, in one particular group of experiments, focal reactions with considerable increase in size of the lesions commonly followed the first injections of tuberculin. This type of response was invariably followed by ultimate increase in size of the lesions considerably beyond that of the controls.

Lesions sometimes began to escape from the suppressive effect of tuberculin therapy as early as the third or fourth injection, but detectable increase in size usually did not begin until 6 or 7 injections had been given. It has been previously shown (O'Grady, 1957) that the later injections in these animals gave rise to reactions which lacked typical delayed components. It might be thought, therefore, that a typical tuberculin reaction is essential if suppression of the lesion is to occur and this would support the belief that the reaction to tuberculin rather than desensitisation is the essential factor. There are, however, two difficulties. In the first place, the change in the responsiveness to tuberculin in these animals is of a special kind in that the progressively accelerated reactions observed were all to the *same* dose. Increasing doses of tuberculin, as employed in tuberculin desensitization, were not given. Secondly, the fall in size of the lesions in response to the first dose of tuberculin was equally well developed and sustained whether a delayed reaction developed to the tuberculin or not (Fig. 1).

It is necessary to emphasise, however, that the failure of a particular tissue to manifest a response to tuberculin does not necessarily mean that some other tissue would not respond if challenged, or that important changes in the responsiveness to tuberculin are not occurring. The fact that the third injection of tuberculin in some of these animals induced a response where previous doses did not, implies an increasing reactivity to tuberculin. In addition, the responses to subsequent doses of tuberculin evolved increasingly rapidly. It appears that it is this period of tuberculin sensitisation which, in these animals, corresponds with the phase of improvement. Once the most rapidly evolving reactions were established, progressive escape from tuberculin therapy occurred (Fig. 2, 3). As a result, the overall control of the lesions obtained by repetition of the same dose of tuberculin was disappointing. Similar escape from treatment with progressive, desensitizing doses of tuberculin has been reported recently in man (Howells and Swithinbank, 1957).

The most important teaching to emerge from the practice of desensitisation was the importance of avoiding focal reactions. Recent studies of tuberculin treatment in man have been complicated by concomitant anti-bacterial treatment and, in the case of tuberculous meningitis, by the fact that focal and local reactions are occurring at the same site. A number of authors (Zieler, 1908 ; Wilkinson, 1926) have distinguished between controlled focal reactions, which are valuable, and uncontrolled reactions which are dangerous. There is no doubt that effective anti-bacterial treatment provides conditions in which focal reactions may be permitted with increased safety. In the present animals, in the absence of anti-bacterial treatment, focal reactions were invariably followed by deterioration.

This type of response was restricted to a group of animals in which other evidence of increased tuberculin sensitivity has been previously noted (O'Grady, 1957).

It is clear that, in groups of these animals, times can be chosen at which the response to treatment offers support for all the major views of the efficacy of tuberculin: that it is valuable, that it is useless, that it is dangerous. With the possibility of all these findings in the same animals, it is not surprising that there has been so little uniformity of opinion about the effects of tuberculin on experimental and human lesions.

SUMMARY

The average size of tuberculous lesions in mouse thighs fell when tuberculin was injected into the other leg. In the majority of groups, repeated tuberculin at 3–4 day intervals produced a progressive fall in size. After 3–7 injections, the lesions escaped from the suppressive effect of tuberculin and returned to the control size. In 2 groups, focal reactions occurred and the lesions in these groups ultimately grew larger than their controls. It is doubted whether desensitization is the important factor in reducing the size of these lesions since the maximal fall in size occurred on the first few injections and escape corresponded with loss of the typical delayed component of the tuberculin response.

I am again greatly indebted to Messrs. D. Burnett, B. Allen, K. Hargreaves, and N. F. Bayley for their able and essential technical assistance.

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THE GROWTH CYCLE OF ECHO VIRUSES IN TISSUE CULTURE: I. MATURATION AND RELEASE OF ECHO VIRUS TYPES 1, 2, 7 AND 11, FROM MONOLAYER CULTURES OF MONKEY KIDNEY CELLS

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A RECENT study by Hsiung and Melnick (1957) demonstrated that the ECHO viruses could be separated into 4 groups by their ability, or otherwise, to form plaques in rhesus monkey kidney cells under agar. The groups depended upon the size and shape of the plaques and the speed with which they first appeared. ECHO virus types 7 (Garnett and Wallace strains), 8 (Bryson) and 12 (Travis) formed a group which produced large circular plaques in 3–4 days. Types 1 (Farouk) and 13 (Hamphill) produced plaques of medium size and irregular boundaries between 4 and 5 days, whereas very small plaques with irregular boundaries first appearing between 5 and 10 days were produced by types 3 (Berardi), 4 (Pesascek), 6 (De Meo), 9 (Quigley), 11 (Gregory) and 14 (Tow). ECHO types 2, 5, and 10 failed to produce plaques after repeated testing.

The differences between groups might reflect the rates of interaction between virus and host cells. We thought that information relating to the nature of these differences might be obtained by a study of the growth cycle of selected members from each group, and the experiments to be described in this paper were undertaken with this objective. In this preliminary study, monolayers of monkey kidney epithelial cells were separately infected with an excess of one ECHO virus belonging to each group. The amounts of virus present in the supernatant fluid bathing the monolayers and within the monolayer itself were assayed at intervals. At the same time monolayers grown on glass coverslips under identical conditions were similarly infected. The rate of development and nature of the cytopathic changes were recorded and these will form the basis of a separate report.

MATERIALS AND METHODS

Viruses

The viruses chosen were as follows: ECHO 1 (Farouk), ECHO 2 (Cornelis), ECHO 7 (Wallace), ECHO 11 (Gregory). Each virus is a member of a different group in relation to its plaque-producing potential. ECHO 2 was in its 13th monkey kidney passage and the remaining viruses had been passed 11 times in monkey kidney. Each had been passed 3 times at limiting dilution and bulk stock had been built up rapidly thereafter. Immediately before use in growth-cycle experiments the infectivity of each virus was titrated in monkey kidney cells.

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Growth medium

This consisted of 93·5 per cent of Hanks' solution, 0·5 per cent lactalbumen hydrolysate, 5 per cent calf serum and 1 per cent sodium bicarbonate (4·4 per cent). This medium was used for the preparation and maintenance on monolayers for growth-curve experiments and for the preparation of stationary tube cultures for infectivity titrations.

Maintenance medium

Earle's saline 90 per cent with 5 per cent calf serum and 5 per cent sodium bicarbonate (4·4 per cent) was used for the maintenance of stationary tube cultures during infectivity titrations.

Phosphate buffered saline (PBS) (Dulbecco, 1954) without calcium or magnesium ions and sodium versenate (1 : 5000) were used for the preparation of second-passage monkey kidney monolayers for growth-curve experiments.

Trypsin (0·2 per cent).—Difco 1 : 250 trypsin was prepared at a concentration of 0·2 per cent in Hanks' salt solution and used for the preparation of primary monkey kidney cultures.

Preparation of primary monkey kidney monolayers

Rhesus monkey kidneys were trypsinised according to the method of Bodian (1956). The cells were distributed either in 12 oz. " medical flat " bottles with screw tops, or in 12 × 100 mm. test tubes with rubber bungs. One or two changes of growth medium were required before confluent monolayers were produced in bottles, usually after 7–10 days' incubation at 36°.

Preparation of secondary monkey kidney monolayers for growth-cycle experiments

Monolayers in 12 oz. bottles were washed twice with PBS and were then suspended by incubating for up to 30 min. in sodium versenate at 36°. The suspended cells were washed by low-speed centrifugation, re-suspended in growth medium and an aliquot was counted in a haemocytometer. A concentration of 3×10^6 cells were added in 8 ml. of growth medium to each 9 cm. petri dish and these were incubated in an atmosphere consisting of 95 per cent air and 5 per cent CO_2 for 3 days.

Infection of monolayers

The procedure adopted was substantially the same as that described by Howes and Melnick (1957). Each monolayer was washed twice in PBS and the infecting virus added in 0·5 ml. of tissue culture fluid containing 4 ID_{50} per cell of the monolayer. Each petri dish was incubated for 1 hr. at 36° in the 5 per cent CO_2/air mixture and was agitated gently every 5 min. to ensure even distribution of the inoculum. After one hour's adsorption the inoculum was removed and each plate was washed rapidly 5 times with 10 ml. PBS at 4°. Of the growth medium at 36° 5 ml. was added and the plate returned to the 5 per cent CO_2/air atmosphere.

Method of harvesting aliquots for infectivity titrations

The supernatant fluid from one petri dish was removed at each hour for 8 hr. and again at 24 hr. Fluids removed 6 hr. or more after the start of each experiment were centrifuged at 3000 r.p.m. for 5 min. in an angle centrifuge to remove any cells which might have become detached from the glass as a result of infection. Each aliquot was then frozen at — 40°.

The monolayer then had 5 ml. of growth medium added and the cells were completely scraped off by a rubber-tipped glass rod. Where cells had been found in the supernatant fluid the packed pellet remaining after centrifugation of the supernatant fluid was returned to the cell fraction, which was then also frozen at — 40°.

Method of disruption of cells to obtain " cell associated " virus

Each cell fraction was thawed and centrifuged to deposit the cells. The packed cells were then separated from the fluid which was retained. The cell fraction was ground in a Ten Broeck grinder for 5 min. before being returned to the separated fluid for infectivity titration. Experiments in the early phase of this work revealed that after one cycle of freezing and thawing and 5 min. grinding complete disruption of the cells occurred.

Virus assays

In an effort to maintain a standardised technique in view of the inability of ECHO virus type 2 to produce plaques, each fraction consisting of supernatant (free virus) and cells (cell associated virus) was assayed for infectivity in tube cultures of monkey kidney cells. Serial dilutions of each fraction were titrated using 5 tubes per dilution and an inoculum of 0·1 ml. per tube. The tubes were read microscopically after 4 days' incubation at 36° and the results graded 0 to 4 + (++++) according to the degree of cytopathic effect present. The infectivity was then calculated in terms of 50 per cent infectious doses (ID_{50}) by the method of Karber (1931).

RESULTS

The results obtained from 2 identical experiments with each virus are shown graphically in Fig. 1–4 and average figures obtained for selected characteristics of the growth cycles of each virus are shown in the table.

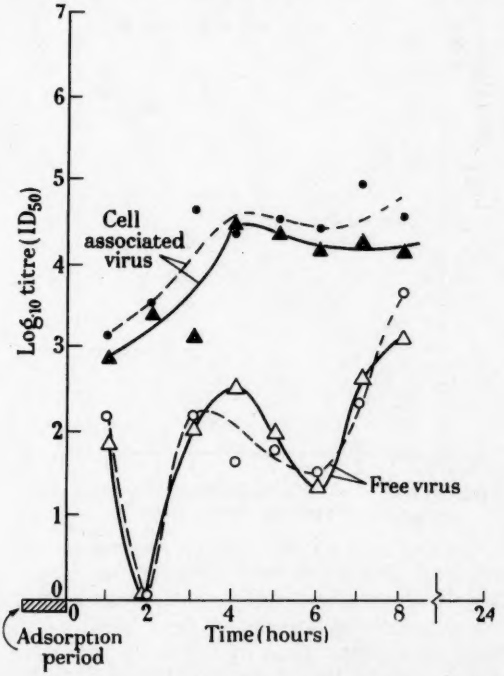

FIG. 1.—Growth cycle of ECHO virus Type 1. The results of two identical experiments are shown.

It will be noted that figures for cell-associated and free virus were obtained at 24 hr. in each case, and that these results have been entered in Fig. 1–4. It was considered, however, that in each instance the curves for cell-associated virus were levelling at about 8 hr. and that this was a probable indication of the end of the first growth cycle. The calculations in the table are based, therefore, on the 8-hr. end-point for each experiment. The average maturation time

(Table) has been defined by Howes and Melnick (1957) as "the time elapsing between inoculation and appearance of 50 per cent of the total mature (infectious) virus produced by the cells", and is a useful figure with which to relate the appearance of mature virus to the growth cycle. The shortest average maturation time was observed with ECHO 1, and this is consistent with the appearance in Fig. 1 of an increase in the cell-associated virus fraction from the second hour of observation. With the other 3 viruses a lag-phase ranging in duration from

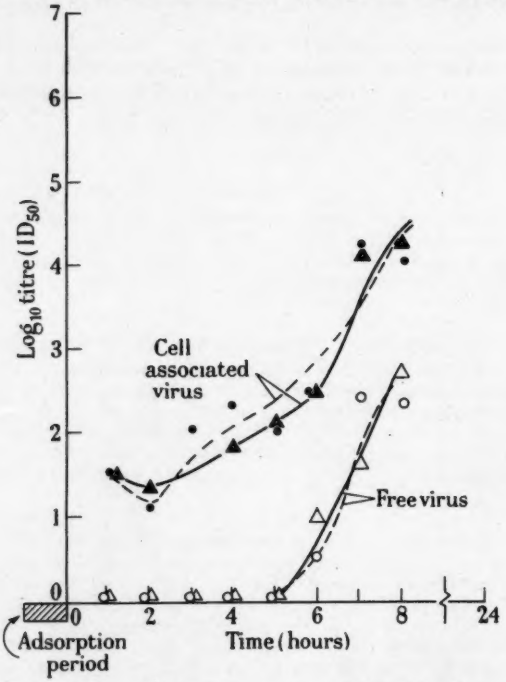

FIG. 2.—Growth cycle of ECHO virus Type 2. The results of two identical experiments are shown.

3 to 5 hr. was evident before cell-associated virus began to increase in amount. Although an average maturation time of 7·3 hr. is recorded in the table for ECHO virus type 2, it will be seen from Fig. 2 that by 8 hr. the curves show least evidence of levelling, which would suggest the end of the cycle, and it is possible that the growth cycle was not fully completed by this stage. It may be, therefore, that a more accurate estimate of the average maturation time would be greater than the 7·3 hr. recorded. However, by 8 hr. the average yield of virus per cell was so much greater than for any of the other viruses that it was felt to be justifiable to retain the 8-hr. end-point for the experiment.

Of interest also is the observation that despite the average maturation time of 3·2 hr. recorded in the table for ECHO 1, by 8 hr. only an average of 0·34 ID_{50} of virus per cell had been produced. This should be contrasted with the

greater average maturation times observed for the other 3 viruses and the associated greater average yields per cell.

The first increases in free virus occur 5 and 3 hr. after cell-associated virus production has commenced with types 1 and 2, whereas the delay in appearance

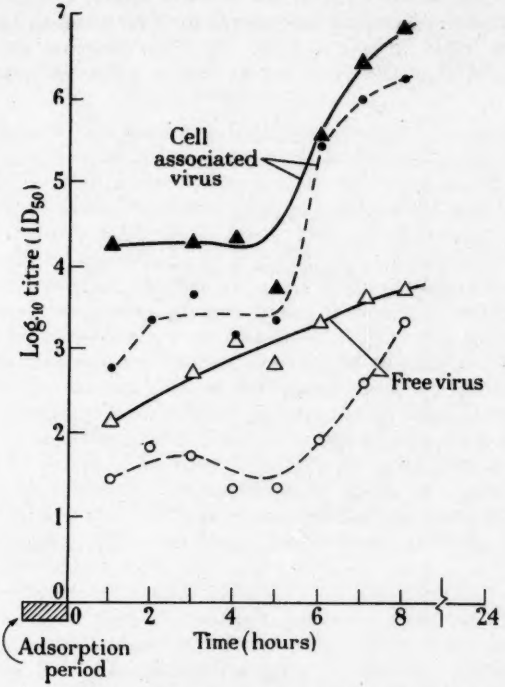

FIG. 3.—Growth cycle of ECHO virus Type 7. The results of two identical experiments are shown.

TABLE.—*Properties of ECHO Virus Types* 1, 2, 7, 11 *Derived from Growth Cycle Experiments in Monkey Kidney Cell Monolayers*

Virus type	Cell-associated virus: First increase (hr.)	Cell-associated virus: Average maturation time (hr.)	Cell-associated virus: Average yield per per cell (ID_{50})	Free virus: First increase (hr.)	Free virus: Proportion free: total virus at average maturation time (per cent)	Plaques: Days to plaque appearance	Plaques: Average diameter of plaque at 10 days after seeding (mm.)
ECHO 2	3	7·3	496	6	1·5	Not produced	—
ECHO 11	5	7·2	142	5·5	2·6	7–10	—*
ECHO 1	2	3·2	0·34	7	1·2	4·5	5·6
ECHO 7	4·5	7·05	59·5	5	0·08	3–4	11·4

* 1·5–2 mm. measured at 13 days.

of free virus is reduced to 0·5 hr. for types 7 and 11. The proportion of free virus produced by the average maturation time in each instance is very low and this is taken to indicate either a delay in the release of cell-associated virus or rapid readsorption to neighbouring cells in the monolayers of virus already released.

Marked fluctuations occur in the curves for free virus in the early stages of both experiments with ECHO 1 (Fig. 1). One possible explanation of this behaviour might be that the virus enters into a phase of unstable equilibrium

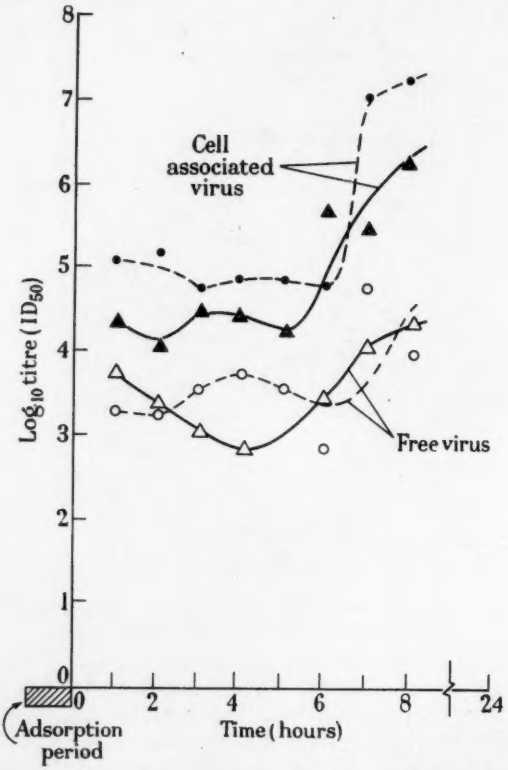

FIG. 4.—Growth cycle of ECHO virus Type 11. The results of two identical experiments are shown.

in its relationship with the cell surface before penetration of the cell wall occurs. The repeated washing at the end of the hour adsorption period may disturb this equilibrium and cause the release into the fluid phase of the culture of numerous particles which then become readsorbed to cells over the next 2 hr. The fluctuations in amount of free virus were also observed in a third experiment where an identical set of conditions was provided but the fractions were harvested half-hourly. The two apparent phases of readsorption of free virus are also of interest

in that during this time a build-up of cell associated virus was occurring in both experiments. Further work is in progress in an attempt to elucidate the underlying reasons for the two readsorption phases.

The information about plaque production in the table is taken from details recorded by Hsiung and Melnick (1957) whose results were obtained with viruses of the same strains as those used in the present series of experiments.

DISCUSSION

From a consideration of these results it is apparent that no direct correlation exists between the results expressed here and the plaque-producing properties of these viruses. If ECHO 1 be omitted from the consideration, however, an inverse relationship exists between the average yield per cell and the average size of plaque produced by the other 3 viruses. Perhaps the most striking anomaly present in these results is seen with ECHO virus type 2 (Table) where the average yield of virus per cell at 8 hr. was 496 ID_{50} and although a relatively small proportion of this amount was present as free virus, it would be reasonable to assume that this would be capable of infecting fresh cells, even under agar, thereby initiating the " chain reaction " which would lead to plaque production. Even if, as suggested by Howes and Melnick (1957), virus released from cells is retained mechanically in an inter-cellular location within the monolayer itself (thus favouring readsorption of newly released virus) it would appear reasonable that ECHO virus type 2 should produce plaques perhaps with even greater speed than any of the other 3 viruses tested. In an identical series of experiments but using poliovirus type 1 Howes and Melnick concluded that between 20 and 40 per cent of the virus produced in the monolayer was released directly into the medium from the free surface of the cells.

Given that no direct correlation appears to exist between the growth cycles as measured here and the plaque producing capacity of the viruses it is of interest to compare the characteristics recorded in the table with those observed by Howes and Melnick (1957) for poliovirus type 1. In their experiments cell-associated virus began to increase after 3 hr. and the growth cycle was virtually complete within 6 hr. The average yield of virus per cell was 100 plaque-forming units and the average maturation time was recorded as 5·4 hr. Increase in free virus commenced shortly after cell associated virus was found to be increasing but by the time 50 per cent of the mature virus had been produced, free virus accounted for only 0·13 per cent of total virus present. The average maturation times and the relatively short growth cycles recorded here might also be compared with the 23 to 26 hr. cycles for adenovirus types 1–4 in HeLa cells reported by Ginsberg (1958).

As with poliovirus type 1 and adenovirus types 1–4 there appears to be a dissociation between the rate of release of free virus from infected cells and the total amount of virus present within the monolayer when ECHO virus types 1, 2, 7 and 11 are considered. These results contrast with the findings of Rubin, Baluda and Hotchin (1955) for Western equine encephalomyelitis virus growing in chicken fibroblasts in culture, when the release of virus occurred at a very rapid rate.

From the evidence produced here it is apparent that the ECHO viruses selected for study in growth curve experiments resemble poliovirus most closely as a

group, but it is also apparent that there are many differences of quite considerable magnitude within the group. These differences between viruses in relation to the growth cycle do not explain the already well known differences in the time of appearance of plaques under agar or of the eventual size of plaque produced in susceptible monkey kidney cells.

SUMMARY

Various characteristic properties of ECHO virus types 1, 2, 7, and 11 have been examined in growth-cycle experiments in monkey kidney cells in an attempt to explain already well known differences in plaque-producing ability. No correlation was found between the numerous characteristics examined and the ability to produce plaques but the viruses studied were found to have growth cycles closely resembling poliovirus.

We wish to express our gratitude to the Secretary of State for Scotland for a personal grant for the salary of one of us and for a grant towards the expenses of this investigation, both made upon the recommendation of the Advisory Committee for Medical Research, and to the Committee of the National Fund for Poliomyelitis Research for a grant towards the expenses of the investigation made on the recommendation of the Medical Research Council.

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THE SYNTHESIS OF PROTEIN IN THE MACROPHAGE AND THE HELA CELL

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IN a previous communication (Harris and Jahnz, 1957) the pattern of incorporation of amino acids into the proteins of the rat connective tissue cell was described. The present communication deals with a similar study on 2 other cells : the macrophage of the rabbit and the HeLa cell. These 3 cell-types cover a wide range of characteristics. They originate from three different species : rat, rabbit and man. The HeLa cell is epithelial in origin ; the connective tissue cell and the macrophage are not. The connective tissue cell and the macrophage are freshly isolated from the tissues for each experiment ; the HeLa cell is taken from a stock culture which has been continuously subcultivated for many years. The connective tissue cell and the HeLa cell multiply *in vitro* ; the macrophage does not multiply, although it remains alive and motile for some days. The connective tissue cell is derived from normal tissue ; the HeLa cell is said to have originated from malignant tissue. If differences in the pattern of amino acid incorporation were common among animal cells, it is reasonable to expect that some evidence of these differences would be found in three cells having such diverse characters.

EXPERIMENTAL

The macrophages were obtained from the peritoneal cavity of the rabbit 6 days after the intraperitoneal injection of liquid paraffin (Lucké, Strumia, Mudd, McCutcheon and Mudd, 1933). The exudate was spun at 220 *g* for 3 min. and the cells resuspended in a medium consisting of 90 per cent of Hanks' solution (Hanks, 1948) and 10 per cent of rabbit serum. Aliquots of this suspension, containing about 5×10^6 cells, were introduced into Carrel flasks of 85 mm. diameter. The macrophages settled on the floor of the flasks and at 37° adhered firmly to the glass. After 30 min. the medium was withdrawn and the cells adherent to the floor of the flasks washed 4 times with physiological saline. Erythrocytes, lymphocytes and dead cells do not adhere to glass, and polymorphonuclear leucocytes adhere poorly under these conditions. Washing the adherent layer of cells thus removes all but living macrophages from the glass. These were maintained in a medium consisting of 50 per cent of rabbit serum and 50 per cent of Hanks' solution. This medium differed from that used in the previous experiments on connective tissue cells only in that rabbit serum was used instead of bovine serum and no cysteine was added. Rabbit serum was used because bovine serum was rather toxic for the macrophages, and cysteine was not added because the macrophages, unlike the connective tissue cells, survived without this supplement.

The HeLa cells were obtained by trypsinization of stock cultures and were grown as a monolayer on the floor of Carrel flasks of 85 mm. diameter. Each flask contained about $2–4 \times 10^6$ cells. During the period when the radioactively labelled compounds were being incorporated the cells were maintained in a medium consisting of 50 per cent of bovine serum and 50 per cent of Hanks' solution. L-cysteine hydrochloride was added to the medium

* In receipt of a personal grant from the British Empire Cancer Campaign.

to make a final concentration of 0·001 M. This medium is thus the same as that used in the previous experiments on connective tissue cells.

All radioactive compounds, except methionine and cystine, were labelled with ^{14}C. Glucose, glycine, L-glutamic acid, L-aspartic acid, L-alanine, L-proline, L-threonine, L-serine, L-leucine, L-isoleucine, L-valine, L-phenylalanine, L-tyrosine, L-arginine and L-lysine were biologically prepared and uniformly labelled. Uniformly ^{14}C labelled histidine, tryptophan, methionine and cystine were not available. Synthetic L-histidine (2-ring-^{14}C), DL-tryptophan (indolyl alanine-3-^{14}C), DL-methionine-^{35}S and DL-cystine-^{35}S were used.

Each labelled amino acid was added to the medium to give a final concentration of 2–4 μc./ml.; the labelled glucose was added to give a final concentration of 7 μc./ml. When labelled glucose was used, glucose was omitted from the Hanks' solution in order to reduce the amount of carrier present. The cells were maintained in the medium containing the labelled compound for 24 hr. After this medium had been withdrawn from the flask, the cells adherent to the floor were washed with 3 changes of physiological saline and then harvested. The methods used for harvesting the cells, preparing hydrolysates of the cell protein and determining the distribution of the radioactivity in the amino acids were described in the previous communication (Harris and Jahnz, 1957).

RESULTS

These are set out in Tables I and II. Comparison of these 2 tables with the table in the previous communication (Harris and Jahnz, 1957) shows that the pattern of amino acid incorporation is essentially the same in all 3 cells. The carbon

TABLE I.—*Incorporation of Radioactively Labelled Amino Acids into the Proteins of the Macrophage*

Radioactivity in each amino acid expressed as a percentage of the total radioactivity in the whole protein hydrolysate

Labelled compound added to medium	Glut.	Asp.	Ala.	Pro.	Thr.	Ser.	Gly.	Leu + Ileu.	Val.	Phe.	Tyr.	Arg.	Lys.	His.	Try.	Met.	Cys.
Glucose	45	21	34	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glutamic cid	78	19	—	2	Tr	Tr	Tr	—	—	—	—	—	—	—	—	—	—
Aspartic acid	30	56	13	Tr	Tr	—	Tr	—	—	—	—	—	—	—	—	—	—
Alanine	Tr	Tr	99	—	—	—	Tr	—	—	—	—	—	—	—	—	—	—
Proline	4	2	—	94	—	—	—	—	—	—	—	—	—	—	—	—	—
Threonine	2	1	Tr	—	94	—	2	—	—	—	—	—	—	—	—	—	—
Serine	—	—	Tr	—	12	43	44	—	—	—	—	—	—	—	—	—	—
Glycine	—	—	—	—	—	1	99	—	—	—	—	—	—	—	—	—	—
Leucine	1	1	—	Tr	—	—	—	97	—	—	—	—	—	—	—	—	—
Iso-leucine	Tr	Tr	—	—	—	—	—	99	—	—	—	—	—	—	—	—	—
Valine	Tr	Tr	—	Tr	—	—	Tr	—	99	—	—	—	—	—	—	—	—
Phenylalanine	Tr	—	—	—	—	—	—	—	—	98	1[1]	—	—	—	—	—	—
Tyrosine	—	—	—	—	—	—	—	—	—	—	100[2]	—	—	—	—	—	—
Arginine	3	1	—	2	—	—	Tr	—	—	—	—	93	—	—	—	—	—
Lysine	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—	—
Histidine[0]	—	—	—	—	—	—	—	—	—	—	—	—	—	100[3]	—	—	—
Tryptophan[0]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[4]	—	—
Methionine-^{35}S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[5]	—
Cystine-^{35}S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[6]

Tr = less than 0·5 per cent. — = no radioactivity present. [0] = not uniformly labelled.

[1] The figure given represents the total radioactivity found in tyrosine and those of its degradation products which appeared on the autoradiograph. [2] All the radioactivity was in tyrosine and its degradation products. [3] All the radioactivity was in histidine and its degradation products. [4] A small amount of radioactivity was found in glycine, but this was regarded as an artefact because some labelled glycine was produced from the labelled tryptophan during hydrolysis. [5] Although radioactivity from methionine-^{35}S appeared in a number of degradation products, there was no radioactivity in cystine or cysteic acid. [6] Radioactivity from cystine-^{35}S appeared mainly in cystine and cysteic acid; no radioactivity from cystine-^{35}S appeared in methionine or methionine sulphone.

skeletons of glutamic acid, aspartic acid, alanine, proline, threonine, serine and glycine are in some measure synthesized by all 3 cells, as evidenced by the presence in these amino acids of carbon derived either from glucose or from other amino acids. The amino acids of this group undergo a good deal of interconversion. The amount of interconversion is, on the whole, greater in the connective tissue cell than in the HeLa cell and greater in the HeLa cell than in the macrophage. This is perhaps related to the rate of protein synthesis in the three cells : the connective tissue cell multiplies more rapidly than the HeLa cell, and the macrophage does not multiply at all. However, the passage of carbon from serine to glycine and from serine to threonine is more marked in the macrophage and the HeLa cell than in the connective tissue cell.

As found with the connective tissue cell, there is no evidence that any of the other amino acids (leucine, isoleucine, valine, phenylalanine, tyrosine, methionine, cystine, arginine, histidine, lysine and tryptophan) is synthesized by the HeLa cell or the macrophage under the present conditions. In all 3 cells some phenylalanine is converted to tyrosine, but tyrosine is not converted to phenylalanine. Apart from this, no interconversion between the amino acids of this second group occurs in any of the cells. In all 3 cells small amounts of carbon from the amino acids which are not synthesized appear in some of the amino acids which are.

TABLE II.—*Incorporation of Radioactively Labelled Amino Acids into the Proteins of the HeLa Cell*

Radioactivity in each amino acid expressed as a percentage of the total radioactivity in the whole protein hydrolysate

Labelled compound added to medium	Glut.	Asp.	Ala.	Pro.	Thr.	Ser.	Gly.	Leu + Ileu.	Val.	Phe.	Tyr.	Arg.	Lys.	His.	Try.	Met.	Cys.
Glucose	36	23	36	Tr	Tr	4	Tr	—	—	—	—	—	—	—	—	—	—
Glutamic acid	66	28	Tr	4	Tr	Tr	Tr	—	—	—	—	—	—	—	—	—	—
Aspartic acid	42	51	4	2	Tr	Tr	Tr	—	—	—	—	—	—	—	—	—	—
Alanine	7	4	87	1	—	Tr	Tr	—	—	—	—	—	—	—	—	—	—
Proline	1	Tr	Tr	98	—	—	—	—	—	—	—	—	—	—	—	—	—
Threonine	Tr	Tr	Tr	—	97	Tr	2	—	—	—	—	—	—	—	—	—	—
Serine	—	—	1	—	22	36	41	—	—	—	—	—	—	—	—	—	—
Glycine	—	—	—	—	—	2	98	—	—	—	—	—	—	—	—	—	—
Leucine	2	1	Tr	Tr	Tr	Tr	Tr	96	—	—	—	—	—	—	—	—	—
Iso-leucine	1	1	Tr	Tr	Tr	Tr	Tr	97	—	—	—	—	—	—	—	—	—
Valine	Tr	Tr	Tr	Tr	—	—	Tr	—	99	—	—	—	—	—	—	—	—
Phenylalanine	—	Tr	—	—	—	—	—	—	—	99	1[1]	—	—	—	—	—	—
Tyrosine	—	—	—	—	—	—	—	—	—	—	100[2]	—	—	—	—	—	—
Arginine	Tr	Tr	—	2	—	—	Tr	—	—	—	—	97	—	—	—	—	—
Lysine	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—	—
Histidine[0]	—	—	—	—	—	—	—	—	—	—	—	—	—	100[3]	—	—	—
Tryptophan[0]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[4]	—	—
Methionine-^{35}S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[5]	—
Cystine-^{35}S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[6]

Tr = less than 0·5 per cent. — = no radioactivity present. [0] = not uniformly labelled.

[1] The figure given represents the total radioactivity found in tyrosine and those of its degradation products which appeared on the autoradiograph. [2] All the radioactivity was in tyrosine and its degradation products. [3] All the radioactivity was in histidine and its degradation products. [4] A small amount of radioactivity was found in glycine, but this was regarded as an artefact because some labelled glycine was produced from the labelled tryptophan during hydrolysis. [5] Although radioactivity from methionine-^{35}S appeared in a number of degradation products, there was no radioactivity in cystine or cysteic acid. [6] Radioactivity from cystine-^{35}S appeared mainly in cystine and cysteic acid ; no radioactivity from cystine-^{35}S appeared in methionine or methionine sulphone.

DISCUSSION

The present investigation shows that in the connective tissue cell, the macrophage and the HeLa cell differences in the pattern of amino acid incorporation are quantitative only. All the amino acids which are synthesized by the connective tissue cell are synthesized also by the macrophage and the HeLa cell, and those which are not synthesized by the connective tissue cell are not synthesized by either of the other cells. Since the three cells have such diverse characters it may be supposed that the pattern of amino acid incorporation which is common to them all is of wide distribution among animal cells. The fact that the HeLa cell after many years of continuous subcultivation has the same pattern of amino acid incorporation as freshly isolated cells implies that if any mutations involving a change in the pattern occurred during this period they did not confer a selective advantage on the mutant under the conditions used for cultivating the cells.

SUMMARY

The synthesis of protein was studied in homogeneous populations of rabbit macrophages and HeLa cells. The pattern of amino acid incorporation in these two cells was found to be essentially the same as that previously found in the rat connective tissue cell. The fact that 3 such different cells have the same pattern of amino acid incorporation suggests that this pattern is widely distributed among animal cells.

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MORPHOLOGICAL AND FUNCTIONAL CHANGES IN CORNEAL ENDOTHELIUM CAUSED BY THE TOXIC EFFECTS OF INFLUENZA AND NEWCASTLE DISEASE VIRUSES*

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VIRUS-INDUCED toxicity has been defined as " the production of lesions in the absence of multiplication adequate to account for them " (Burnet, 1955). During the past 15 or 20 years this term has come to be associated specifically with a group of more or less related pathological states experimentally induced by the inoculation of high concentrations of a suitable virus into animals. The observed lesions commonly include haemorrhage, vascular congestion and oedema singly or in combination depending upon the route of injection of the virus (Rake and Jones, 1944; Henle and Henle, 1946; Ginsberg, 1951). Such observations suggest that alterations in the capillary endothelium may present a factor of general significance in virus-induced toxicity. The possibility that this tissue represents the primary " target " of the virus remains to be tested.

The introduction of large amounts of certain viral agents into the anterior chamber of the rabbit eye elicits the development of corneal opacity without a concomitant rise in virus titre (Evans and Rickard, 1945; Evans and Bolin, 1946; Bolin, Anderson, and Leymaster, 1950). It seems likely that this opacity is the result of a movement of fluids into the corneal stroma in response to an alteration in the permeability of the endothelial lining of the anterior chamber. The unique architecture of the anterior chamber, around which a single layer of endothelial cells forms an almost continuous membrane, provides a favourable situation for observing the effect of toxic concentrations of virus upon these cells.

It is the purpose of this paper to present evidence which indicates that certain viruses are indeed capable of reacting directly with corneal endothelium in such a fashion as to destroy the physiological and morphological integrity of this membrane.

MATERIALS AND METHODS

Virus preparations

The Beaudette strain of Newcastle disease virus (NDV) obtained from Dr. Seymour Levine and Influenza A virus (PR-8 strain) obtained from the American Type Culture Collection were employed. Undiluted allantoic fluid suspensions were used to elicit the toxic reactions.

Tests of endothelial permeability

The rabbits used in these studies were injected with 0·2 ml. of NDV into the right eye and an equal amount of normal allantoic fluid into the opposite eye. Eighteen to 24 hr.

* Aided by financial assistance from a contract with the Office of Naval Research and by the State of Washington Fund for Biological and Medical Research.

later a unilateral toxic reaction was well developed. At this time ^{131}I-labelled human albumin obtained from Abbott Laboratories, Chicago, Illinois was injected either intravenously or directly into the anterior chamber of both the toxic and control eyes. At intervals thereafter the animals were sacrificed and samples of aqueous humour, blood and cornea were assayed for ^{131}I content with a well-type scintillation counter. Changes in endothelial permeability were estimated by a comparison of the distribution of labelled material in the normal and the toxic eye of each animal.

Virus and antibody titrations

NDV infectivity was assayed by the plaque-count technique on monolayers of cells derived from chick embryos (Dulbecco, 1952). Influenza infectivity was determined by egg titration, the 50 per cent infectious titre being calculated by the method of Reed and Muench (1938). Haemagglutination (HA) titrations were carried out by a modification of the Salk pattern test designed to minimize the effect of HA inhibitors often present in biological materials (Granoff, 1955). Anti-NDV antibody concentrations are expressed as haemagglutination inhibition (HI) units (Salk, 1944) or as serum equivalents; one serum equivalent is defined as that concentration of antiserum which reduced the infectivity of a given amount of virus to e^{-1} or 37 per cent (Dulbecco, Vogt and Strickland, 1956). The effect of non-specific inhibitors was eliminated by the use of heat inactivated serum (56° for 30 min.) in all titrations for neutralizing antibody.

Corneal preparations

At appropriate intervals following injection of the virus into the anterior chamber the animals were killed and the corneal endothelium was prepared for cytological examination by gently scraping the posterior surface of the cornea with a blunt scalpel and spreading the detached endothelial cells on a glass slide. These preparations were immediately fixed in methanol and stained by Wolbach's variation of the Giemsa stain. Whereas this method resulted in good preparations for evaluating individual cell changes it gave little information concerning the integrity of the entire endothelial lining and thus no quantitative data on the extent of endothelial damage resulting from the toxic reactions.

To follow the initiation, spread, and extent of the endothelial lesions, the endothelial cell borders were stained while still on the excised cornea by a 30-sec. exposure to a 0·5 per cent solution of $AgNO_3$. To prevent the interference of naturally occurring chlorides, the corneas were rinsed briefly in isotonic sucrose both before and after staining. They were then fixed in methanol. By careful dissection the endothelial layer was teased free from the underlying cornea and small fragments were transferred to glass slides. This permitted the application of various stains (Hansen's iron trioxyhaematin, Giemsa) to the same endothelial preparation for more precise cytological studies.

RESULTS

The morphology of the toxic reaction

The normal endothelium, when stained with silver nitrate, revealed a regular mosaic pattern of polyhedral cells (Fig. 1). The application of a nuclear stain showed a single, very large, bean-shaped nucleus within each of these cells (Fig. 2). Occasionally 2 or 3 nuclei were observed within the apparent limits of a single cell.

Within 2 hr. following the injection of $1—2 \times 10^8$ plaque-forming particles (PFP) of NDV the normal mosaic pattern of the endothelial cells was altered. This change was characterized by a progressive failure of the silver nitrate to delineate cell boundaries, resulting in what appeared to be a fusion of adjacent cells (Fig. 3). Initially only widely scattered pairs of adjacent cells were involved; however, by the fourth hour numerous areas could be found which appeared to represent the fusion of 6 or more cells (Fig. 4). Superimposed nuclear stains at this stage revealed apparently normal nuclei within these areas. The obliteration

of silver staining boundaries progressed until, 6–8 hr. following the introduction of virus, only an occasional line of deposited silver could be observed on the endothelial surface (Fig. 5 and 6). Up to this time preparations stained with Giemsa or trioxyhaematein revealed little evidence of cellular damage. Subsequent to this, however, the development of cytoplasmic granularity (Fig. 7 and 8) and vacuolization in similarly stained preparations were interpreted as an early sign of cytonecrosis. Eighteen to 24 hr. following the introduction of virus the posterior surface of the cornea was usually devoid of endothelial cells and appeared to consist of Descemet's membrane littered with debris and leucocytes.

Ordinarily a faint corneal opacity became grossly apparent within 6 hr. This reached a maximum within 24 hr. (Fig. 9) and, after a variable period, a slow return to normal transparency often occurred. Depending upon the amount of virus injected, any degree of reaction between complete irreversible opacity and slight cloudiness could be obtained.

Inasmuch as it is well established that an intact endothelium is necessary for the maintenance of corneal transparency (Thomas, 1955), recovery of previously opaque eyes was considered to be evidence of endothelial regeneration. This was substantiated by the observation that every recovered eye suitably examined was found to possess a regenerated corneal endothelium.

Microscopic examination of corneas exhibiting only mild toxic reactions revealed the simultaneous presence of normal, degenerating and regenerating cells. On the other hand, examination of corneas with severe reactions demonstrated what appeared to be a complete loss of corneal endothelium prior to the establishment of the regenerative process.

Regenerating cells were characteristically larger and more irregular in shape than normal endothelial cells (Fig. 10 and 11). It appeared that these cells were incapable of restoring the normal physiological status to the cornea since corneal transparency returned only after such cells reverted to an essentially normal appearance. It was frequently noted that the morphological appearance of the cells directly underlying opaque patches in otherwise transparent eyes was typical of regenerating endothelium whereas the endothelium underlying the transparent areas of the same cornea was normal in appearance.

Frequently the opacity associated with severe toxic reactions proved to be irreversible. Examination of these eyes indicated that the posterior surface of the cornea had become permanently repopulated by cells which were extremely irregular in shape, resembling those seen in Fig. 10.

The morphological changes accompanying the reaction induced by influenza virus were identical to those just described for NDV but developed more slowly.

The fate of NDV in the anterior chamber of the rabbit eye

An accurate appraisal of the fate of virus introduced into the anterior chamber of the eye was difficult. Several factors contributed in varying degrees to the loss of virus from this area. First among these was the variable amount of virus lost through back-leakage of the inoculum following withdrawal of the needle from the eye. Another factor of importance was the increasing ability of the aqueous humour to reduce both the haemagglutinating activity and the infectivity of the virus. This became evident within 10 min. following the trauma associated with intraocular injection and was presumably due to the influx of non-specific virus inhibitory components from the serum (Karzon, 1956). The escape of virus in

conjunction with the rapid circulation of the aqueous humour (Kinsey, Grant and Cogan, 1942*a*; Kinsey, Livingood and Curtis, 1942*b*) was probably of less importance. The observation that convalescent animals rarely possessed even small amounts of specific antiviral antibody makes it doubtful that appreciable amounts of virus escaped into the circulation.

Duplicate experiments were undertaken to study the fate of NDV in the rabbit eye. In each, several animals were injected intraocularly with $1{\cdot}5 \times 10^8$ plaque-forming particles (PFP) of virus in a volume of 0·2 ml. At appropriate intervals the animals were sacrificed and 0·1 ml. of aqueous humour was withdrawn, diluted and assayed for plaque-forming particles. In addition, the *in*

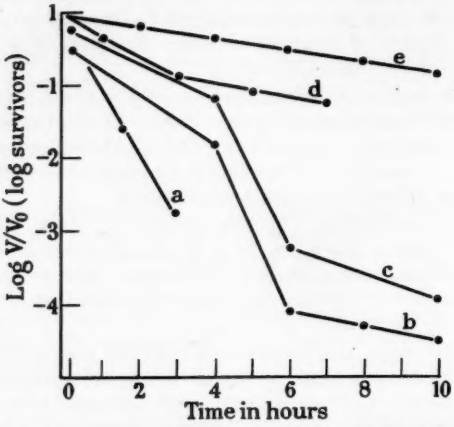


FIG. 12.—The *in vivo* and *in vitro* decline of NDV infectivity. (*a*) *In vitro* inactivation of NDV in aqueous humour withdrawn 4 hr. after corneal puncture. (*b*) and (*c*) *In vivo* decline of NDV in aqueous humour (2 experiments). (*d*) *In vitro* inactivation of NDV in aqueous humour. (*e*) *In vitro* inactivation of NDV in a balanced salt solution.

vitro inactivation of NDV at 37° in a balanced salt solution, normal aqueous humour and aqueous humour withdrawn 4 hr. after traumatizing the cornea by a single needle puncture was followed. The results of these assays are plotted in Fig. 12. The rapid drop in the *in vivo* titres observed during the first 10 min. was largely due to leakage which, however, soon ceased to be a factor. The increased rate of virus inactivation between 4 and 6 hr. following the injection of virus was attributed to the accumulation of non-specific virus inhibitory material in the aqueous humour. In support of this concept it is interesting to note that the rate of inactivation of NDV *in vitro* by aqueous humour removed 4 hr. after traumatizing the cornea paralleled that of the *in vivo* tests between the fourth and sixth hours. After the maximum effect of non-specific inhibitor had been attained the rate of virus disappearance decreased and, from the sixth hour onward, closely paralleled the rate of thermal inactivation of NDV in a balanced salt solution.

As has been reported previously for influenza virus (Evans and Rickard, 1945), no evidence of virus multiplication could be detected. Examination of aqueous humour and endothelial lysates (prepared by alternate freezing and thawing) at

intervals up to 30 hr. following injection of the virus failed to reveal any evidence of an increase in either non-infectious haemagglutinin (NIH) or infectious virus (PFP). It must be remembered, however, that the concentration of non-specific virus inhibitory substances in the aqueous humour may have been sufficient to mask any signs of multiplication.

Alterations in endothelial permeability following the development of corneal opacity

Rabbits exhibiting fully developed unilateral toxic reactions, induced by the injection of NDV into the right eye, were employed in a series of experiments in which equal amounts of ^{131}I-labelled albumin were introduced into the anterior chambers of both the toxic and opposite control eyes of the same animals. At intervals of 30 min., 3 hr. and 24 hr. following the introduction of the labelled material, rabbits were killed and the concentrations of ^{131}I in aqueous humour, blood and cornea were determined. It was readily apparent that the corneas of the toxic eyes accumulated more radioactive material than did those of the opposite control eyes (Table). This was presumably due to the destruction of the endothelial lining of the toxic corneas resulting in the removal of a barrier to the passage of albumin from the aqueous humour into the corneal stroma. With regard to this, it is interesting to note that the water content of the toxic corneas averaged 88 per cent by weight as compared to 75 per cent for the opposite control corneas. At the same time it was noted that the loss of labelled material from the aqueous humour of the toxic eyes was less rapid than that from the control eyes ; a factor which undoubtedly contributed somewhat to the elevated levels of isotope found in the toxic corneas. It would appear that the normal drainage of aqueous humour from the anterior chamber was impaired.

TABLE.—*The Distribution of ^{131}I-Labelled Albumin After Injection into the Anterior Chambers of Rabbit Eyes Previously Treated with NDV*

		Cornea			Aqueous humour		
		Specific activity†		Ratio§ Toxic/	Activity‡		Ratio Toxic/
Animal	Interval*	Toxic	Control	Control	Toxic	Control	Control
1	30 min.	316	33	9·6	1515	354	4·3
2		421	67	6·3	1286	1159	1·1
3		248	30	8·4	1465	328	4·5
4		640	137	4·7	2247	2834	0·8
5	3 hr.	287	16	17·9	1326	10	132·6
6		292	7	41·7	822	33	24·9
7	24 hr.	42	12	3·5	35	3	11·7
8		147	47	3·1	38	4	9·5

† Specific activity = counts/min./mg. dry wgt.
‡ Activity = counts/min./mg. wet wgt.
* Time from injection of labelled albumin to test for activity. Albumin was injected 24 to 48 hr. after virus. All toxic eyes showed fully developed reactions.
§ Numbers are numerical values of the ratio of the numbers in the 2 columns to the left.

The effect of a toxic corneal reaction on passage of protein from the blood stream into the eye was also examined. With respect to this it was noted that following the intravenous injection of labelled albumin the aqueous humour of

toxic eyes accumulated radioactive material more rapidly than did that of the control eyes. It is not unlikely that the mild inflammatory response often evident at this time was responsible for this concentration of labelled material in the anterior chamber of the toxic eye. Despite the fact that the blood levels of isotope following intravenous injection of labelled material were from 10 to 100 times greater than those achieved following intraocular injection, corneal radioactivity averaged less than one-tenth of that of the intraocularly injected animals. Since there appeared to be no relationship between blood and corneal levels of labelled material it seems probable that the movement of labelled albumin from aqueous humour into corneal stroma was, for the most part, direct.

Challenge of the regenerated endothelial membrane with toxic amounts of homologous virus

A transitory period of refractoriness to challenge with the homologous virus following a sublethal toxic reaction has been shown to occur in several systems (Wagner, 1952 ; Dougherty and Groupé, 1957*a*). It was not surprising, therefore, that the challenge of a partially or completely regenerated corneal endothelium with the same virus used to elicit the original reaction often failed to initiate a second reaction. In 7 rabbits challenged in this manner with NDV there was neither gross nor microscopic evidence of a toxic reaction. This ability to resist challenge remained complete for at least 2 weeks following the initiation of the original toxic reaction. Challenge of different animals after this period revealed an increasing degree of endothelial damage and opacity until ultimately the eye regained its normal susceptibility. Although variable, this return to normality occurred most typically at 3 to 5 weeks following the original injection of virus.

The sera of both resistant and normal rabbits were shown to contain a heat-stable haemagglutination inhibitor (Hirst, 1942) and a heat-labile inhibitor of virus infectivity (Karzon, 1956) both of which are non-specific in nature. Only occasionally could the sera of resistant animals be shown to contain even very low levels (1 in 4) of specific neutralizing antibody.

Aqueous humour removed from both normal and resistant rabbit eyes just prior to injection of virus showed no evidence of either specific or non-specific virus inhibitory activity. Following the trauma of intraocular injection, however, the presence of appreciable levels of heat-stable haemagglutination inhibitor (40 to 80 HI units) and heat-labile virus inhibitor could be demonstrated. It is important to note that the presence of similar inhibitor levels produced by traumatizing normal eyes was not sufficient to prevent a toxic reaction following the injection of standard amounts of NDV.

In order to assess the contribution of specific circulating antibody to the refractory state, 3 normal rabbits were inoculated twice weekly by alternating intravenous and intramuscular injections of allantoic fluid NDV for a total of 6 weeks. During this period the serum haemagglutination-inhibition titres rose from an average value of 1 in 128 to 1 in 1536. This latter value corresponds to 1100 serum equivalents per ml. Seven days following the last immunizing injection these animals were injected in both eyes with NDV, as were 2 normal control animals of the same size and age. Within 24 hr. every eye, including those of the controls, exhibited a marked toxic reaction.

As an alternate approach to this problem 7 normal animals were injected with NDV in the right eye only. Within 24 hr. 6 of these animals showed gross toxic

reactions in the treated eyes. Seventeen days later recovery of transparency was complete in 2 of the rabbits, partial in 3 and had not occurred in one animal. At this time both the treated and untreated (right and left) eyes were challenged with NDV. As was expected, 6 of the 7 treated eyes were resistant to challenge and no further signs of toxicity could be detected either grossly or microscopically following sacrifice ; however, all 7 of the untreated control eyes showed a gross toxic reaction within 24 hr.

The results of these 2 experiments are interpreted as evidence that the resistance to homologous challenge shown by eyes following a toxic reaction is not due to humoural antibody.

DISCUSSION

Clarke and Fox (1948), working with typhus rickettsia in mice, first suggested that a seriously altered vascular permeability was the major factor underlying the toxic reaction. The frequency with which haemorrhage and oedema have been associated with virtually every type of virus-induced toxic reaction (Cox, 1953) adds to the probability that endothelial tissue represents a " primary target " in reactions of this type. The data presented in this paper indicate that certain viruses are indeed capable of acting more or less directly upon at least one type of " endothelial " cell in such a way as to destroy the functional integrity of the membrane which these cells compose. Further studies will be required to determine to what extent the mechanisms responsible for the corneal reaction are of general significance.

The cell-virus interaction resulting in destruction of the endothelium may well represent that of an abortive infection. This concept of the nature of viral toxicity was first advanced by Schlesinger (1950), who observed the production of NIH accompanying the neurotoxic action of influenza virus. More recently Henle, Girardi and Henle (1955) observed a similar effect *in vitro* following the treatment of HeLa cells with influenza virus. In view of our failure to demonstrate any degree of virus multiplication, it is important to bear in mind that recent evidence indicates the virus synthetic sequence may be blocked or diverted prior to the appearance of either NIH or complement fixing antigen (Prince and Ginsberg, 1957) ; therefore, the absence of readily detectable virus components does not rule out infection of this type.

It is necessary to consider the possibility that endothelial destruction is the result of a surface reaction similar to the haemolysis of erythrocytes, a well known property of certain of the myxoviruses (Burnet and Lind, 1950 ; Chu and Morgan, 1950). This does not seem likely, however, in view of the fact that influenza virus has no such haemolytic ability and yet is fully as active as NDV in producing the corneal reaction.

Because of the uncertainty regarding the structural and functional basis of the silver staining reaction, the progressive changes demonstrated early in the toxic reaction by utilizing this technique are difficult to evaluate. The fact that the silver stain reveals early morphological changes not made apparent by other techniques underlines the desirability of determining the nature of this reaction. This may well provide an important clue to the nature of the functional damage caused by the virus.

The establishment of a state refractory to further challenge coincided with the recovery of the toxic eye. Refractory states have also been reported in other

more or less similar systems (Wagner, 1952; Dougherty and Groupé, 1957*a*). Unlike the more transient immunity associated with these other virus-induced toxic reactions (Khoobyarian and Walker, 1957), the resistance of the newly regenerated corneal endothelium persists for periods in excess of 2 weeks. Preliminary experiments indicate that this state of immunity is not related to the production of humoural antibody. This is in agreement with the conclusions reached by other workers utilizing a different system (Dougherty and Groupé, 1957*b*). In view of these observations at least 3 different mechanisms must be considered as possible bases for this immunity: (*a*) An autointerference mechanism may be operative although the immunity seems unduly prolonged for this; (*b*) some sort of local antibody response may occur (Witmer, 1955); (*c*) the endothelial cells may be resistant as a result of physiological changes associated with regeneration and recovery of normal ocular function.

SUMMARY

Corneal toxicity, induced by the intraocular injection of either Newcastle disease virus (NDV) or influenza A (PR-8) virus, is associated with a progressive destruction of the corneal endothelium. By employing a silver staining technique it was possible to observe very early morphological changes in this membrane, not made apparent by other methods. Studies utilizing ^{131}I labelled human albumin indicated that the endothelial changes produced an altered exchange relationship between the corneal stroma and aqueous humour; as a result, corneal oedema and opacity developed. Although the cell-virus interaction resulted in cellular destruction, there was no evidence for virus multiplication as determined by infectivity and haemagglutination assay.

The regeneration of the corneal endothelium was followed by a regression of corneal oedema and opacity. It could be shown that regenerating or recently regenerated endothelial cells were refractory to challenge with toxic doses of the

EXPLANATION OF PLATES

Fig. 1, 3, 4, 5, 6 and 10 represent corneal endothelium stained with silver nitrate. Both silver nitrate and iron haematoxylin were used to stain the corneal endothelium shown in Fig. 2, 7, 8 and 11.

FIG. 1.—Normal rabbit corneal endothelium. × 200.

FIG. 2.—The same endothelium as shown in Fig. 1 stained to show nuclei. × 475.

FIG. 3.—Corneal endothelium 2 hr. following the injection of NDV into the anterior chamber. × 200.

FIG. 4.—Corneal endothelium 4 hr. following injection of NDV. × 200.

FIG. 5.—Corneal endothelium 6 hr. following injection of NDV. × 200.

FIG. 6.—Corneal endothelium 8 hr. following injection of NDV. × 200.

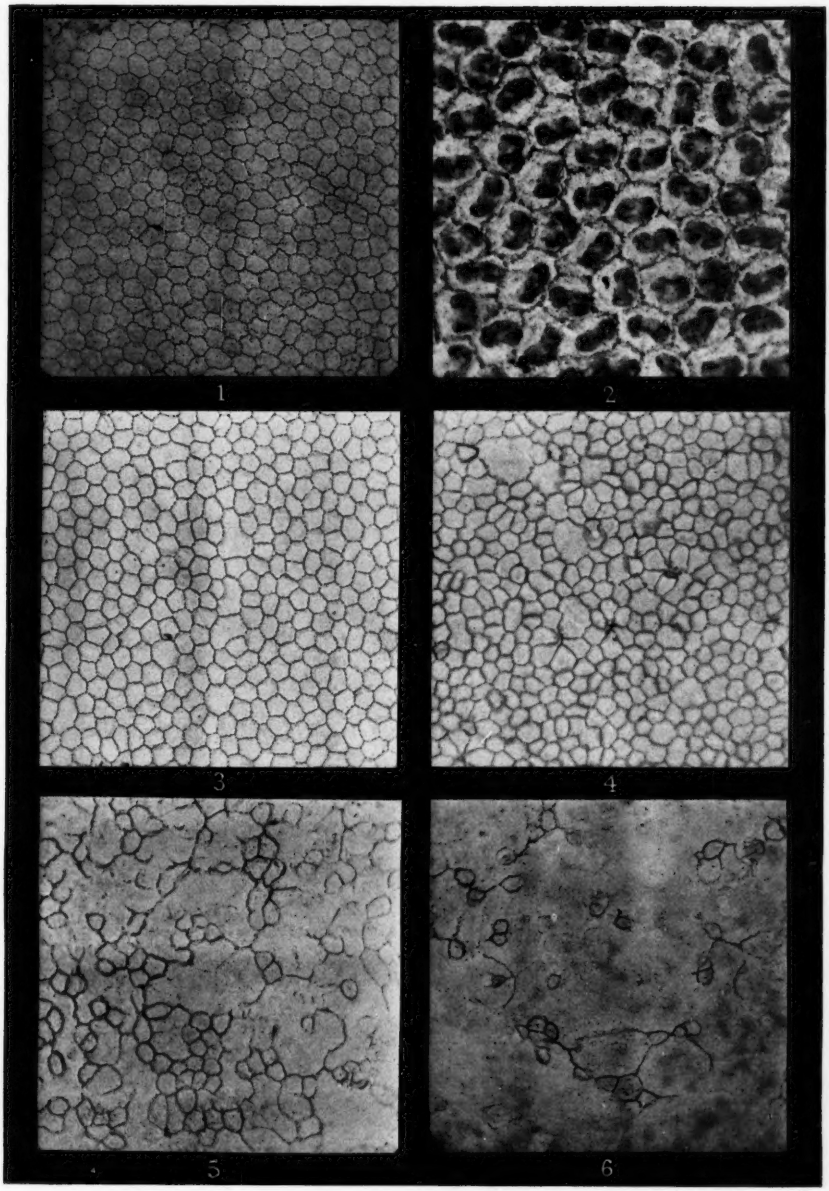
FIG. 7.—The same endothelium as shown in Fig. 5 (6 hr. post injection) showing early nuclear changes. × 475.

FIG. 8.—The same endothelium as shown in Fig. 6 (8 hr. post injection) showing increased granularity and nuclear changes. × 475.

FIG. 9.—Corneal opacity following the injection of NDV into the anterior chamber of the rabbit's eye.

FIG. 10.—Regenerating corneal endothelium 7 days after injection with NDV. Note the large size of these cells. × 200.

FIG. 11.—Regenerating corneal endothelium 14 days after injection with NDV. The typical mosaic is beginning to reform. × 200.



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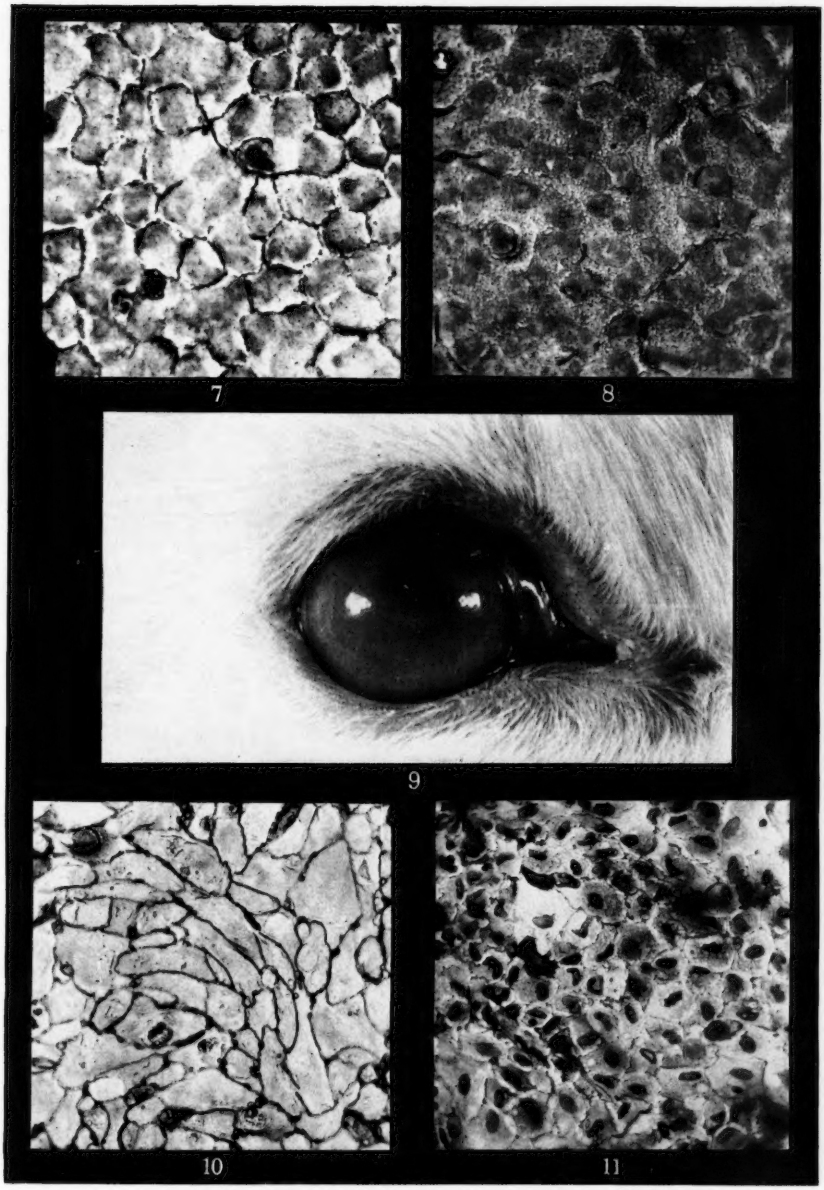

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homologous virus for periods up to several weeks. This transitory immunity appeared to be unrelated to humoural factors.

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EXPERIMENTAL PYELONEPHRITIS: THE INFLUENCE OF TEMPORARY AND PERMANENT URETERIC OBSTRUCTION ON THE LOCALIZATION OF BACTERIA

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ALTHOUGH pyelonephritis is a serious and common illness, the major problems of pathogenesis remain unsolved. The route by which bacteria reach the kidney and the factors which determine how, and where, they localise are imperfectly understood. A few genera of bacteria will localise in the healthy kidney following intravenous injection (Navasquez, 1950; Gorrill, 1952) but the majority including *Bacterium coli* will not.

Lepper (1921) showed that a kidney, from which the flow of urine was obstructed by ureteric ligation, developed pyelonephritis when *Bact. coli* was injected intravenously, whilst the kidney on the other side remained normal. This finding has since been repeatedly confirmed. Furthermore, Mallory, Crane and Edwards (1940) claimed that removal of the ligature 4 or 5 days after injection of bacteria would halt the infection. Recently, quantitative studies on the rate of multiplication of *Bact. coli* in the obstructed kidney have been made by Gorrill (1956) using the rabbit and by Guze and Beeson (1956) in the rat. Gorrill (1956) sampled portions of cortex and medulla separately and found that bacteria multiplied immediately in the medulla whereas in the cortex no increase in bacterial count, was found for 90 min. Guze and Beeson (1956), performing whole organ counts, observed that no increased trapping of bacteria occurred in the obstructed kidney compared with the unobstructed one, and that numerical increase in bacteria was delayed for 4 to 6 hr. The present study was undertaken to investigate the effects of permanent and temporary ureteric occlusion on renal localisation and multiplication of *Bact. coli* in the rat.

METHODS AND MATERIALS

The organism used for all experiments was a *Bact. coli* isolated from a patient with acute pyelonephritis. To facilitate subsequent identification of the organism it was made resistant to 100 μg. per ml. streptomycin and a detailed study made of the biochemical properties. The organism was stored by freeze-drying. For a batch of experiments an ampoule was opened and a culture kept on an agar slope at 4°. For a particular experiment the organism was grown in tryptone broth for 18 hr., the suspension centrifuged and resuspended in phosphate buffer.

Estimation of opacity was carried out in the Spekker absorptiometer and the suspension adjusted to contain approximately 400×10^6 per ml. viable organisms by use of a previously prepared calibration curve relating viable count to opacity. An aliquot of this suspension was retained and an accurate viable count made.

The technique of injection of organisms was to expose the jugular vein under light ether anaesthesia, inject 0·5 ml. of bacterial suspension (approx. 200×10^6 organisms) and close the wound with a continuous suture.

For enumeration of bacteria in the blood and in the kidneys, the rat was again lightly anaesthetised with ether, and the abdomen and thorax opened by a ventral incision. Blood was taken from the heart by puncture with a sterile Pasteur pipette and 0·1 ml. measured into 9·9 ml. buffered saline. Serial dilutions were made and viable counts carried out by flooding nutrient agar plates, which had been dried at 37° for 4 hr., with 0·5 ml. of each dilution. Duplicate plates were used for each count. When dry the plates were incubated overnight and counted next day.

The kidneys were removed and homogenised in 9 ml. buffered saline using glass tissue grinders. Viable counts were determined by the technique used for the blood.

Female albino Wistar rats (Glaxo) weighing between 180 and 200 g. were divided into 4 experimental groups.

Group I was composed of 40 rats in which there was no interference with the ureter. These were injected with *Bact. coli* and 4 rats killed at timed intervals (Table I), viable counts being carried out on blood and kidneys as already described. Before removal, the left kidney was perfused with 80 to 100 ml. of sterile physiological saline via the abdominal aorta with the right renal artery clamped.

Group II consisted of 76 rats in which the left ureter was exposed through a lumbar incision and tied off by means of a thread 1 cm. distal to the pelvi-ureteric junction. Organisms were injected 30 to 45 min. later. Subgroups of rats were killed after the same time intervals as Group I (Table II) and viable counts made on the kidneys and blood. Perfusion of the left kidney was not attempted because a preliminary experiment showed that following mobilisation in tying off the left ureter, perfect perfusion was impossible.

Group III was made up of 31 rats in which ureteric occlusion was released after 6 hr. After exposing the kidney through a lumbar incision a length of thick white silk was looped round the upper part of the ureter and after passing the silk through the lumbar muscles on one side of the incision, the latter was sutured using black thread. The ends of the white silk were then tightened and finally tied, thus drawing the ureter up against the inside of the lumbar muscles and causing it to be kinked. The skin was approximated with 3 stitches. After 30 to 45 min. the organisms were injected as before. Six hours after injection, under light ether anaesthesia, the skin stitches were removed and the white silk pulled out with the ureter falling back into its usual position. This was a modification of a method used successfully in the rabbit (Heptinstall and Gorrill, 1955). Viable counts, without perfusion of the left kidney, were made at intervals of from 1 day to 4 weeks as in Group II.

Group IV.—Twenty-one rats were treated in exactly the same way as Group III except that the obstruction to the ureter was left for 24 hr. before release. Either 4 or 5 rats were bled and killed at intervals of 2 days to 4 weeks (Table IV).

RESULTS

The main findings are shown in Tables I, II, III and IV.

Group I (Table I).—Blood culture showed a very high level at 15 min. falling rapidly until at 6 hr. it was virtually sterile.

The bacterial counts in the right non-perfused kidney were initially higher than in the left perfused kidney but at 6 hr. they were almost equal and from 1 day onwards were similar. The results show quite clearly that proliferation of organisms in non-obstructed kidneys does not occur and that by 7 to 14 days sterility of the kidneys is the rule.

Group II.—In this group of rats with permanent occlusion the blood counts were similar initially to those found in Group I. Although there were slightly higher counts at 6 and 24 hr. there was virtual sterility at 2 days. However, when the kidney counts are compared, those on the obstructed side showed no gross disproportion until between 2 and 6 hr. after injection of organisms. Maximal counts were reached between 2 and 4 days and this fitted in well with the macroscopic appearance of abscesses by the 2nd day. At the 14th day 2 of the 4 obstructed kidneys showed lower counts, this being accounted for by destruction of parenchyma

by necrosis (pelvic contents were discarded before homogenising the kidney so as to confine counts to bacteria within the renal parenchyma). Counts in the non-obstructed kidney showed close agreement with those of Group I.

Group III.—In this group ureteric obstruction was released after 6 hr. The number of organisms recovered from the blood was substantially the same as that of the two previous groups. No proliferation of organisms was noted in the unobstructed kidney except for 1 rat which showed the anomalous count of 590,000 organisms at 4 weeks, for which no explanation was obvious. After 24 hr., 4 out of 6 rats showed a high count similar to Group II. At 2 days only 2 out of 6 rats had a count of the order of those in Group II, and in 4 animals the count had fallen below one million organisms. Later counts showed a progressive reduction in bacterial population making it clear that the lesion was healing. No macroscopic evidence of abscess formation was seen in any of the kidneys and no scarred areas indicative of healed abscesses were found. In all cases the ureter which had been temporarily occluded had returned to its usual position and there was no hydronephrosis.

Group IV.—In this group the blood was not examined until 48 hr. after injection of the organisms and at this time and subsequently it was sterile. No proliferation of organisms was found in the non-obstructed kidney and although positive cultures persisted longer than in any of the other groups, high counts were never found. At 2 days, in the obstructed kidney of 3 out of 4 rats, counts of the same magnitude as those of Group II were found and small cortical abscesses were seen. High counts were still present at the 4th day, but at 7 days counts were substantially lower than in Group II, only 1 rat having a high count and this

TABLE I.—*To Show* Bact. coli *Content of Blood and Right and Left Kidneys in Group with No Interference with Either Ureter (Group I)*

Time of killing	Blood	Right kidney (not perfused)	Left kidney (perfused with saline)	Time of killing	Blood	Right kidney (not perfused)	Left kidney (perfused with saline)
15 min.	186,000	44,400	10,600	24 hr.	0	1,900	1,000
	310,000	55,400	15,700		0	1,700	1,600
	258,000	23,200	11,100		0	600	1,200
	89,000	14,100	8,400		0	5,300	4,500
30 min.	36,000	44,800	22,800	2 days	0	5,700	3,300
	26,000	45,400	27,200		0	9,900	18,000
	24,000	20,400	5,000		0	7,100	5,100
	21,000	14,500	9,300		0	24,800	16,600
60 min.	0	10,600	3,900	4 days	0	80	10
	0	11,300	4,400		500	500	0
	7,000	9,500	5,100		0	12,800	10,700
	4,000	9,700	4,200		0	3,000	3,000
120 min.	2,000	17,400	5,900	7 days	0	5,600	80
	8,000	18,800	5,800		0	0	0
	3,000	10,600	3,000		0	600	1,000
	0	4,200	1,000		0	0	0
6 hr.	0	9,900	3,000	14 days	0	0	0
	1,000	4,200	3,500		0	0	40
	0	8,800	4,400		0	0	0
	0	3,400	300		0	0	0

TABLE II.—*To Show* Bact. coli *Content of Blood and Right and Left Kidneys in Group with Permanent Occlusion of Left Ureter (Group II)*

Time of killing	Blood	Right kidney	Left kidney (ureter tied)	Time of killing	Blood	Right kidney	Left kidney (ureter tied)
15 min.	832,000	44,600	49,200	24 hr.	0	6,800	4,660,000
	158,000	11,600	15,500		0	3,400	66,000,000
	239,000	19,800	27,800		7,000	1,900	77,000,000
	281,000	22,000	23,600		6,000	1,100	324,000,000
	221,000	28,000	27,800		0	5,400	29,800
	260,000	31,000	28,000		2,000	6,800	22,400,000
					2,000	4,400	6,420,000
30 min.	21,000	6,000	12,000		6,000	6,500	17,000,000
	27,000	7,000	15,000		0	3,800	700
	8,000	10,500	18,200		0	1,700	5,400
	6,000	11,000	14,000				
	16,000	9,000	26,400	2 days	1,000	12,800	212,000,000
	36,000	8,200	13,000		1,000	2,500	1,050,000,000
	36,000	7,500	17,600		0	2,500	289,000,000
	12,000	9,000	16,000		0	10,600	162,000,000
					0	2,000	420,000,000
60 min.	10,000	17,000	12,000		0	8,000	38,000,000
	10,000	10,800	11,300		0	18,200	80,000,000
	6,000	8,200	10,700		0	3,200	46,000,000
	3,000	9,600	12,200				
	6,000	14,000	19,000	4 days	0	8,500	0
	6,000	7,600	12,600		0	500	2,164,000,000
	5,000	6,800	8,900		0	3,600	32,000,000
	4,000	6,200	7,200		0	3,300	28,000,000
					0	700	216,000,000
120 min.	2,000	3,400	5,000		1,000	2,400	250,000,000
	3,000	5,800	10,200		0	5,100	90,000,000
	2,000	12,000	14,800		0	700	150,000,000
	2,000	9,400	10,300				
	0	4,500	7,700	7 days	0	5,800	24,000,000
	3,000	5,900	8,200		0	0	28,000,000
	0	2,200	14,000		0	5,400	58,000,000
	0	2,300	16,000		0	1,000	40,000,000
					0	10,800	12,000,000
6 hr.	0	1,400	1,412,000		0	400	28,000,000
	1,000	880	82,800		0	20	12,000,000
	2,000	5,100	248,000		0	500	394,000,000
	1,000	4,000	230,000				
	0	2,800	1,182,000	14 days	0	0	2,000,000
	3,000	900	1,432,000		0	700	340,000,000
	4,000	3,500	814,000		0	0	200,000
	1,000	6,500	78,000		0	1,500	440,000,000

animal had an incomplete stricture at the site of the temporary ligature. Thereafter the number of bacteria declined and by the 14th day only 1 out of 4 animals had a count of over a million and macroscopically flat yellow areas were seen in the cortex. By 4 weeks depressed scars were seen in the cortex.

DISCUSSION

The results given in Table I show the inability of *Bact. coli* to produce progressive lesions in the healthy kidney. Although bacteria can be found in the kidneys for up to 7 days following intravenous injection, there is no tendency for them to multiply. It was hoped that perfusion with saline of the left kidney would give a more reliable estimate of the number of organisms actually in the kidney parenchyma by eliminating bacteria contained in the blood circulating through

TABLE III.—*To Show* Bact. coli *Content of Blood and Right and Left Kidneys with 6 hr. Occlusion of Left Ureter (Group III)*

Time of killing	Blood	Right kidney	Left kidney (ureter occluded for 6 hr.)	Time of killing	Blood	Right kidney	Left kidney (ureter occluded for 6 hr.)
1 day	0	100	5,000	7 days	0	8,600	79,200
	0	3,100	4,100,000		0	2,000	100
	0	9,000	126,000,000		0	2,100	30,000
	0	2,100	<1,000		0	45	50
	5,000	4,600	73,000,000		0	0	100
	0	6,000	2,500,000				
				14 days	0	6,900	20
2 days	0	4,600	4,500		0	1,600	400
	1,000	7,200	1,800		0	20	250,000
	0	2,300	112,800		0	0	0
	0	4,100	28,880,000				
	0	22,000	69,400	4 wk.	0	590,000*	198,000
	0	8,600	1,320,000		0	0	0
					0	0	0
4 days	2,000	12,800	1,000		0	0	3,700
	6,000	2,700	202,000				
	1,000	3,200	380,000				
	0	6,700	2,000				
	0	6,200	5,200				
	0	9,100	5,400				

* Anomalous count referred to in text.

TABLE IV.—*To Show* Bact. coli *Content of Blood and Right and Left Kidneys with 24 hr. Occlusion of Left Ureter (Group IV)*

Time of killing	Blood	Right kidney	Left kidney (ureter occluded for 24 hr.)	Time of killing	Blood	Right kidney	Left kidney (ureter occluded for 24 hr.)
2 days	0	2,300	340,000,000	14 days	0	0	40,000
	0	14,000	42,000,000		0	5,400	124,000
	0	2,700	402,000,000		0	1,100	4,000
	0	9,200	100,000		0	5,400	3,200,000
4 days	0	2,400	18,000,000	4 wk.	0	0	326,000
	0	5,700	100,000,000		0	7,100	99,000
	0	1,800	1,720,000,000		0	0	0
	0	15,900	6,800,000		0	10	22,800
7 days	0	700	1,800,000				
	0	6,900	700,000,000				
	0	1,100	830,000				
	0	1,000	20,000				
	0	0	101,000				

the kidney. However, accepting that the bacterial uptake is the same in right and left kidneys when there is no interference with the ureter (Guze and Beeson, 1956), when perfusion was carried out, the counts found early in the experiment were much lower than those expected from the theoretical calculation which assumed the blood contained in the kidney vessels to be 14 per cent of the kidney volume (Weaver, McCarver and Swan, 1956). Therefore it would seem that perfusion removes bacteria other than those which are simply passing through the kidney in the blood stream. After 24 hr., by which time the blood culture is

negative, no significant difference was found between perfused and non-perfused kidneys.

In Group II the ureter on the left side was occluded. Following intravenous injection of log. phase cultures no great increase of bacteria was obvious before 120 min. but at 6 hr. it was clear that rapid multiplication of bacteria had occurred, abscesses being seen by the 2nd day (Table II). These findings compare closely with those of Guze and Beeson (1956) but differ from those of Gorrill (1956) who, working with rabbits, found that organisms which localised in the medulla of the kidney entered the log. phase at once, and those in the cortex after a delay of only 2 hr. Rapid multiplication of organisms was found also by Braude, Shapiro and Siemienski (1955) who used renal massage to cause *Bact. coli* to localise in the kidney.

A preliminary experiment had shown that release of the ureter after 2 days accompanied by removal of the opposite kidney resulted in the death of only 1 out of 9 animals. From this experiment it was assumed that if obstruction of the ureter was released after 24 hr., or less, function of the obstructed kidney would return. Because considerable multiplication of bacteria was apparent on the obstructed side at the end of 6 hr., it was decided to observe the effect on the bacterial count, of release of ureteric obstruction after 6 hr. (Table III, Group III) and 24 hr. (Table IV, Group IV). It was found that in Group III high counts on the obstructed side were usually reached after 24 hr. but after 2 days the counts showed a definite diminution: in Group IV the decline was delayed until the 4th day. The results in both these groups contrast with Group II, where obstruction was permanent, in which high counts were regularly maintained up to 7 days and until the 14th day in 3 out of 4 animals. These differences were well shown by the macroscopical appearance of the kidneys. In Group II extensive abscess formation took place whereas, in Group IV abscess production was far less marked, and by 4 weeks healing had occurred producing depressed scars in the cortex. In Group III no macroscopic evidence of abscess formation was seen in any of the kidneys and no scarred areas were found at 4 weeks.

The precise way in which obstruction to urinary outflow increases susceptibility to infection is not clear. Navasquez (1950) found that coagulase positive staphylococci when injected intravenously produced renal abscesses without the need for prior ligation of the ureter. These abscesses healed with scar formation and in a subsequent experiment Navasquez (1956) showed that these scarred kidneys were susceptible to infection when *Bact. coli* was given intravenously. Navasquez (1956) attributes this ability of *Bact. coli* to localise in such kidneys to the internal hydronephrosis associated with tubular deformities such as those described by Oliver (1945, 1950) in scarred kidneys. This was considered to cause a raised local tissue pressure with consequent slowing of the circulation and trapping of bacteria (Navasquez, 1956).

Asher and Sokol (1941) had previously postulated that stasis of the renal circulation was responsible for the establishment of infection in the obstructed kidney. These explanations are in keeping with the observation of Lucas (1908) that ureteric obstruction slows the blood flow through the kidney and that of Brewer (1911) that partial obstruction of the renal artery or vein resulted in the production of renal abscesses in the dog when *Bact. coli* was injected intravenously. If in fact occlusion of the ureter does slow the renal circulation, our findings do not support the concept of increased trapping of organisms. There was no increased

initial deposition of organisms in the obstructed kidney and although they were injected in the log phase, there was no great increase until between 2 and 6 hr. Beeson, Rocha and Guze (1957) produced scarring within the renal medulla by an electric cautery and rendered that part of the kidney whose tubules passed through the scarred area susceptible to infection. They concluded, however, that the ability of *Bact. coli* to localise was not due to interference with the circulation.

The common factor in most of the procedures used to produce experimental pyelonephritis is tubular obstruction. The obvious explanation for proliferation of organisms in such kidneys is that tubular blockage permits growth of organisms in the obstructed nephrons. The inability of bacteria to pass through the glomerular filter in the normal kidney as shown by Helmholz and Millikin (1925) and Asher and Sokol (1941) has always been considered to militate against such an explanation. However, Mallory, Crane and Edwards (1940) showed that in the obstructed kidney one of the earliest cortical lesions is a glomerulitis followed by glomerular abscess formation, so that in the obstructed kidney organisms soon reach the tubules. In addition to this, interstitial abscesses form in relation to the peritubular capillaries with eventual rupture into tubules. The absence of free drainage in the obstructed kidney is likely to perpetuate an infective process and the establishment of such drainage by release of ureteric occlusion might be expected to promote healing. This may be one of the reasons for the healing in rats of Group IV although other factors may be involved. The recent experiment of Guze and Beeson (1958) clearly demonstrates the importance of the degree of obstruction in the production of pyelonephritis. Partial ureteric occlusion produced by irradiation of the ureter in both the rat and rabbit, although producing hydronephrosis did not render the kidney vulnerable to bacterial infection after intravenous injection of *Bact. coli*.

SUMMARY

Bacterial counts of blood and kidney were carried out at different time intervals up to 4 weeks on rats injected intravenously with *Bact. coli*.

One group had no interference with the ureter, another had the left ureter permanently obstructed while two other groups had temporary occlusion of the left ureter for 6 hr. and 24 hr.

Progressive proliferation of organisms in the kidney occurred in the group with the permanent obstruction but in the groups with temporary occlusion after an initial proliferation, the organism counts decreased.

The findings lend no support to the concept that there is an initial increased deposition of organisms in the obstructed kidney which initiates the infective process.

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INTERFERENCE BY ENDOTOXIC POLYSACCHARIDES WITH INACTIVATION OF COLIPHAGE BY HUMAN SERUM

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VARIOUS polysaccharide complexes are now known to combine with properdin *in vitro* and to alter properdin levels *in vivo*. This was shown first with zymosan (Pillemer, Blum, Lepow, Ross, Todd and Wardlaw, 1954) from yeast cell wall, later with polysaccharide endotoxins of Gram-negative bacteria (Pillemer, Schoenberg, Blum and Wurz, 1955; Landy and Pillemer, 1956*a*) and with analogous polysaccharide materials derived from mammalian and plant tissues (Pillemer, Landy and Shear, 1957). The only substances known, at present, to have such affinity for this serum protein are polysaccharide-containing complexes. Although most of the materials which we have thus far found to interact with the properdin system also possess endotoxic properties, other, non-endotoxic, polysaccharides of natural origin are also known to interact with properdin (Pillemer *et al.*, 1955).

Several procedures have been developed for measuring the biological behaviour of the properdin system. These include: inactivation of the third component of complement (Pillemer, Blum, Lepow, Wurz and Todd, 1956); bactericidal action against Gram-negative bacteria (Wardlaw and Pillemer, 1956); lysis of PNH (Paroxysmal Nocturnal Haemoglobinuria) erythrocytes (Hinz, Jordan and Pillemer, 1956); and inactivation of coliphage (Van Vunakis, Barlow and Levine, 1956). In the foregoing systems, the alterations produced in properdin and/or complement are measured. One polysaccharide (zymosan) has been employed in studies of the mechanisms but, here too, the emphasis has been upon the changes produced in the third component of complement.

The question arose as to whether properdin interaction would provide a useful method for study *in vitro* of endotoxic polysaccharides. Whether or not properdin is a single substance or whether its activity represents the effect of a number of related substances is presently being investigated in various laboratories. As concerns the neutralization of coliphage by human serum, evidence has just appeared (Cowan, 1958) which indicates that properdin is not necessarily a single entity. Whatever be the ultimate outcome of his general problem it is nevertheless possible to obtain useful comparisons of the potency of a series of endotoxic polysaccharides in blocking the phage-neutralizing action of human serum. The above-mentioned procedures vary in their suitability for such a purpose. Thus, the zymosan procedure requires rather large amounts of serum and of polysaccharide; hence it does not provide a sufficiently sensitive measure of polysaccharide. The bactericidal method is markedly affected by the wide-

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spread distribution of antibodies to Gram-negative bacteria. The interaction of properdin with PNH erythrocytes would offer promise if such cells were readily obtainable. On the other hand, the procedure based on inactivation of coliphage by human serum offers the advantages associated with classical phage techniques ; furthermore, this system requires only small amounts of properdin. If it were found that small amounts of polysaccharide could reverse the inactivation of phage by properdin, this situation could then be employed for measuring this activity of polysaccharides.

It was found that the neutralization of T2r$^+$ coliphage by human serum could indeed be annulled by polysaccharides. In the course of exploring the factors influencing this interaction it became apparent that, under appropriate conditions of test, abrogation of the antiviral action of the properdin system was linearly related to dose of polysaccharide. Details of this study are presented together with the results obtained in the use of the method in measurement of this activity of polysaccharides *in vitro*.

MATERIALS AND METHODS

Culture media.—Nutrient broth medium was prepared from dehydrated Difco Bacto nutrient broth supplemented with 5 g. of sodium chloride per litre. The plating agar contained, per litre, 10 g. Difco Bacto agar, 13 g. Difco Bacto tryptone, 8 g. sodium chloride, 2 g. sodium citrate and 1·3 g. glucose. Each litre of suspending agar contained 6 g. Difco Bacto agar, 10 g. Difco Bacto tryptone, 8 g. sodium chloride, 2 g. sodium citrate and 3 g. glucose. For assay, 9·5 ml. of liquefied suspending agar was tubed (22 × 175 mm.) and kept in a water-bath at 42° until used in plating.

Veronal buffer.—To a litre of hot distilled water, 4·7 g. 5,5-diethyl barbituric acid and 2·9 g. of its sodium salt were added. After the solution had cooled, 2·5 g. sodium bicarbonate and 10·7 g. sodium chloride were added ; the volume was then brought to 2 litres with distilled water. The final pH of the buffer, as measured with a glass electrode, was 7·5.

Culture.—The stock culture of *Bacterium coli* B, maintained on agar, was stored at 4°. For each assay, a tube of nutrient broth was inoculated with the stock culture and incubated, with shaking, at 37° ; after 4 to 6 hr. it was diluted with 4 volumes of nutrient broth and incubated for an additional hour. This served as the bacterial inoculum for the suspending agar.

Phage.—The T2r$^+$ coliphage, prepared and purified by the method of Herriott and Barlow (1952), was kindly supplied by the latter in a suspension containing 1×10^{13} particles per ml. This concentrated suspension, when diluted with 0·88 per cent saline containing 10 μg. gelatin per ml., provided a stock suspension containing 2×10^{11} particles per ml. For daily use, appropriate dilutions of the virus suspension, stored at 4°, were prepared in the Veronal buffer supplemented with 0·01 M Mg^{++}.

Polysaccharides.—These were endotoxic preparations derived from various Gram-negative bacteria, plants and mammalian tissues. The bacterial polysaccharides were of varied generic origin ; some represented different methods of isolation (Landy and Pillemer, 1956*b*). The preparation and biological properties of the polysaccharides derived from plant and mammalian tissues have been reported previously (Belkin, Perrault and Shear, 1955 ; Perrault and Shear, 1955 ; Landy and Shear, 1957). Many of the pilot experiments in the present work, particularly those concerned with technique, were carried out with the polysaccharide preparation from *Bryonia* root. This product of plant origin was selected because of the quantity on hand and because it had been characterized as a glucose-polymer with only trace amounts of nitrogen and phosphorous (Shear, unpublished).

Sera.—Blood drawn from healthy individuals was allowed to clot by standing at room temperature for 2 hr. The clear serum obtained on centrifugation was promptly transferred to pyrex ampoules which were flame-sealed and stored at − 50°. Serum pools (mixtures of equal volumes of 50 or more individual sera) served as reference sera for daily assays. Immediately on preparation they were distributed in 1, 2 or 5 ml. quantities and stored in the frozen state. They were thawed as required ; unused portions were discarded.

Properdin reagents.—Purified human properdin, human RP and rabbit anti-human properdin serum were kindly supplied by the late Dr. Louis Pillemer.

Terminology.—Since fresh serum is known to neutralize coliphage but not to destroy its infectivity we have avoided the use of the term viricidal. Instead, the action of fresh serum on virus is designated as " antiviral ", " inactivating " or " neutralizing ". For convenience these terms are used interchangeably. In referring to the effect of polysaccharides in counteracting the antiviral action of fresh serum, the terms " annul " " block ", " reverse ", " inhibit" and " abrogate " are employed interchangeably.

EXPERIMENTAL

The techniques utilized in the present study were, unless otherwise designated, those described by Barlow, Van Vunakis, and Levine (1958*a*) who presented evidence showing that complement, Mg^{++}, and properdin were the factors in human serum required for inactivation of coliphage. They demonstrated that the limiting component in serum is properdin. Inasmuch as it had previously been established that many polysaccharide complexes interact with properdin *in vitro* (Pillemer, Landy and Shear, 1957), it was expected that the phage-properdin interaction might also be counteracted by polysaccharides. In that case, since this coliphage system was responsive to small increments of properdin, it was anticipated that it would be especially sensitive to reversal by small amounts of polysaccharides which interact with properdin.

Properdin requirements for phage inactivation

Many experiments were first carried out to ascertain whether this system was, in fact, properdin dependent. Taken as a whole, they showed that properdin was indeed required for inactivation of coliphage by fresh serum. These experiments, confirming the findings of Barlow, Van Vunakis and Levine (1958*a*), are summarized in Table I. It is thus clear that purified properdin restored antiviral activity to an RP otherwise inactive, and that the addition of antiproperdin abolished the activity of fresh serum. The view that properdin plays an essential role in the inactivation of coliphage is supported by these findings.

TABLE I.—*Inactivation of Coliphage by Human Serum : Evidence for Properdin Requirement*

Treatment of serum	Subsequent antiviral activity	Remarks
Heat-inactivation (30 min. at 56°)	None	Except for occasional serum with heat-stable inhibitor.
Absorption with zymosan	,,	According to procedure employed to prepare RP.
Zymosan absorbed serum + purified properdin	Restored	Activity dependent on quantity of properdin.
Addition of rabbit anti-properdin serum	None	Function of dose of antiserum.
Addition of polysaccharides previously shown to complex with properdin	,,	Function of dose of polysaccharide.

Selection of sera

Ideally the serum selected should be free of heat-stable inhibitors, such as specific antibodies, and should supply an excess of complement and an appropriate level of properdin. In the early stages of this investigation, serum from a single

donor proved satisfactory in these respects. However other sera, tested in the same way, were found to vary widely as regards the effect of polysaccharide on their antiviral activity even though the quantity of serum employed was adjusted to provide equivalent virus neutralizing activity. These observations indicated that, in addition to the components of complement which are known to be required for complexing of properdin and polysaccharides, there are other, still unrecognized, factors involved in this interaction. These findings are consistent with the observations of Pillemer (1956) that only 20 per cent of sera treated with zymosan yield satisfactory RP, *i.e.*, sera completely free of properdin following treatment with zymosan.

It was possible to obtain sera of appropriate virus neutralizing content, whose activity was annulled by polysaccharide as a function of dose, by examining a sufficient number and selecting from these the few that were satisfactory. The problem of obtaining suitable individual sera in this way is formidable. This difficulty was obviated by pooling large numbers of normal sera. The reproducibility of data obtained with 3 such serum pools, as regards sensitivity to inhibition by 5 polysaccharide preparations, is shown in Table II. These data suggest that whatever the nature of the additional factors required for complexing, and the unpredictability of their levels in individual sera, the pooling of a sufficient number of sera yields satisfactory uniform results.

TABLE II.—*Uniformity of Polysaccharide Assays with Different Serum Pools*

Source of polysaccharide	Quantity of polysaccharide required to abolish 50 per cent of the antiviral activity of 3 serum pools		
	A	B	C
	(μg.)	(μg.)	(μg.)
Human liver	0·75	0·72	0·73
Mouse carcinoma	10·5	9·8	10·7
Bryonia	15·0	13·0	12·5
Chromobacterium prodigiosum	14·5	15·0	14·5
Salmonella enteriditis	120	125	130

Effect of prior incubation of serum on its antiviral action

To facilitate the interaction of serum with polysaccharide, the mixture is incubated at 37° for 1 hr. As a check on the effect of this incubation on the antiviral activity of serum itself, specimens of individual and of pooled sera were treated in this way and then assayed for antiviral activity in parallel with unheated aliquots. From Fig. 1 it is seen that the incubated sera gave steeper slopes of viral inactivation than did the unheated. Thus treatment of serum for 1 hr. at 37°, *before* the addition of phage, resulted in considerable augmentation of its antiviral action. All 3 serum pools showed this effect of "preincubation" (Fig. 1, A, B, C); however, only some of the individual sera did (Fig. 1, D).

These experiments were carried out to determine whether the additional hour of incubation would reduce the antiviral activity of the serum itself. Unexpectedly, the effect obtained was in the opposite direction. It is thus clear that serum itself must be treated in the same way as the serum-polysaccharide mixture if it is to serve as an appropriate control.

Effect of polysaccharide preparations on the antiviral activity of fresh serum

On the basis of our earlier findings (Pillemer *et al.*, 1957) it was anticipated that these polysaccharides would also interfere with, or block, the antiviral action of the properdin system. This was found to be the case and in addition, it was soon evident that the inhibitory action of polysaccharides was greatly influenced by the conditions of test.

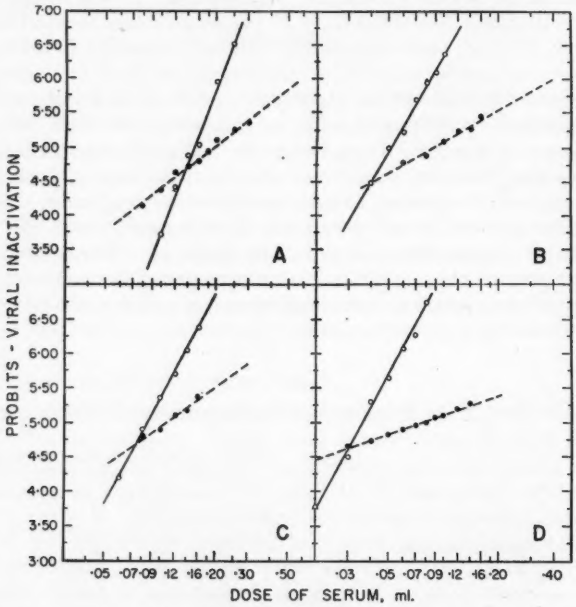

FIG. 1.—Augmentation of antiviral action of human serum. ○———○ Serum incubated for 1 hr. at 37° prior to intesection with phage. ●———● Untreated serum interacted with phage.

Time-temperature relationships.—Omission of the prior incubation of polysaccharide with serum yielded results which contrasted markedly with those obtained when the polysaccharide-serum mixture was first incubated (Fig. 2). When prior incubation with serum was omitted, the slope obtained by plotting degree of inhibition against dose was shallow; *i.e.*, the inhibitory action of polysaccharide was poorly related to dose. As the prior incubation period was increased, the inhibitory effect of the polysaccharide became more pronounced, and reached a maximum at 1 hr.; this remained unchanged on more prolonged incubation. The inhibition by polysaccharide is temperature dependent. When the incubation was carried out at 17° or at 4° little or no effect was obtained. For this interaction to proceed to completion it was essential that polysaccharide and serum be incubated at 37° for 1 hr. The phage then provided the indicator system for disclosing the quantity of residual properdin still available for antiviral action.

Polysaccharide complexes, such as the one derived from *Bryonia* root, may be

sedimented by high speed centrifugation. Under conditions in which treatment of fresh serum with polysaccharide did not alter antiviral activity, *i.e.*, incubation at 17° or 4°, high speed centrifugation had no effect upon the activity. This was expected; on the other hand, when treatment with polysaccharide abolished antiviral activity, *i.e.*, incubation at 37°, high speed centrifugation might well have resulted in restoration of activity; however, this did not occur.

Effect of concentration of serum.—Throughout this work, the reference serum was employed in an amount sufficient to inactivate approximately 85 per cent of the coliphage. This level of serum was selected because it afforded the broadest range for inhibition by polysaccharide and yet it provided a residual amount of unaffected phage to serve as a convenient base line for calculation. This amount

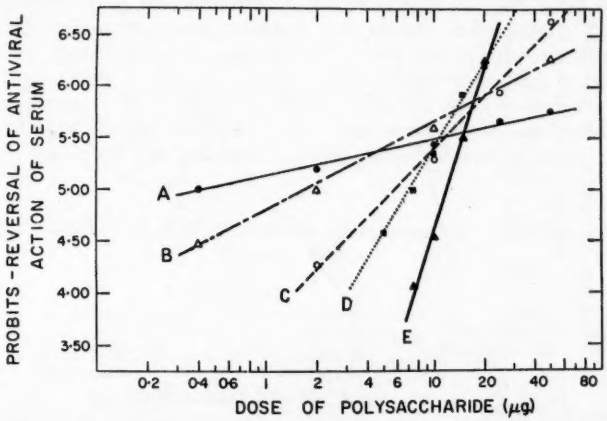

FIG. 2.—Inhibition by polysaccharide of the antiviral action of human serum. Influence of incubation time at 37°: A = 0 min., B = 20 min., C = 30 min., D = 40 min., E = 60 min.

of serum provided a test system of high sensitivity to inhibition by polysaccharide. Over most of this dose range (15 to 85 per cent inactivation of phage) probits of per cent inactivation plotted against log serum dose gave a straight line; hence the antiviral action of fresh serum was directly related to the quantity used. With a constant dose of serum, the inhibitory action of polysaccharide likewise was linear. Fig. 3 shows that as the quantity of serum was increased, the amount of polysaccharide required to counteract the antiviral activity also increased. As the quantity of serum increased, the slope of inhibition by polysaccharide became less steep and therefore provided a less sensitive measure of inhibition by polysaccharide. The centre slope is representative of the test conditions selected for use in this work. In our experience, the amount of serum which inactivates about 85 per cent of the phage provides a satisfactory steep slope of dose-response.

Inactivation of complement by polysaccharides.—In addition to properdin and Mg^{++}, the recognized components of complement are known to be required for the inactivation of coliphage (Barlow, Van Vunakis and Levine, 1958*a*). In the annulment by polysaccharide of this effect several mechanisms are conceivable: it might specifically complex with properdin and render this substance unavailable for inactivation of phage; the polysaccharide-properdin complex might specifi-

cally inactivate one or more of the components of complement ; or the polysaccharide might be non-specifically anti-complementary and inactivate one or more of the components of complement required for the reaction. The end result of each of these reactions would be the same, namely, abolition of the serum action on coliphage. To avoid the non-specific loss of complement components, an amount of serum was employed which was found to provide an excess. Moreover, the polysaccharides routinely were tested for anti-complementary activity at a level equal to the largest amount used in the assay. A considerable number of polysaccharides, particularly those of mammalian origin, appeared to be anti-complementary when they were incubated with serum at 37°, in that there was a

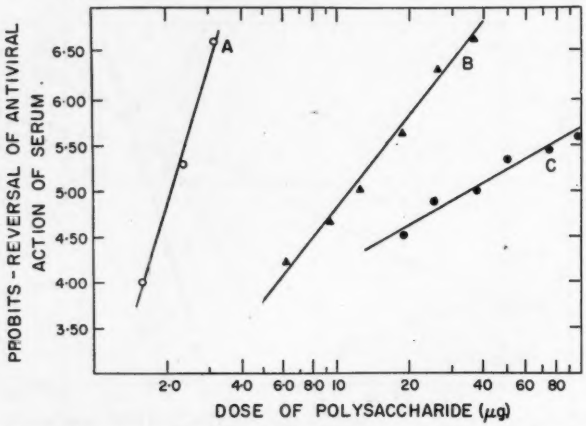

FIG. 3.—Inhibition by polysaccharide of the antiviral action of human serum. Influence of quantity of serum. A = 0·04 ml., B = 0·08 ml., C = 0·12 ml.

loss of haemolytic activity. However, experiments such as the following provided evidence that this is not simply a non-specific anti-complementary effect.

Fresh human serum treated with anti-properdin no longer suffered a loss of haemolytic activity upon treatment with polysaccharide ; in other experiments, incubation of a human RP with these polysaccharides likewise resulted in no loss of haemolytic activity. Thus, in the absence of properdin, inactivation of haemolytic complement by these polysaccharides did not occur.

Incubation of fresh human serum and polysaccharide for 1 hr. at 37°, followed by the addition of sufficient guinea-pig complement to restore the full haemolytic activity of the serum did not restore antiviral activity. Thus the inhibition by polysaccharide of the antiviral action of the properdin system is not to be ascribed to the unavailability of complement as a consequence of its destruction by polysaccharide ; haemolytic complement is lost, but only in consequence of an interaction requiring properdin.

Effect of concentration of polysaccharide.—For measurement of polysaccharide activity, the following procedure was adopted. Fifty or more fresh, normal sera were pooled to serve as the source of properdin and complement. The quantity used in the test was the amount found, in preliminary experiments, to inactivate

approximately 85 per cent of the standard dose of phage (1×10^4). This quantity of serum, in 0·2 ml. of saline, was dispensed into tubes (14 × 125 mm.). Each test included 2 sets of controls, in quadruplicate, on the input of phage and on the antiviral activity of the reference serum. Four or more progressive dilutions (in Veronal buffer) of the polysaccharide, selected to annul between 15 and 85 per cent of the antiviral action of the reference serum, were prepared in a volume of 0·3 ml. This was added to the 0·2 ml. of serum and the mixture was allowed to interact for 1 hr. at 37°. The tube were dosed with 1×10^4 particles of coliphage contained in 0·5 ml. of Veronal buffer containing 0·01 M Mg^{++}; the system was then incubated for an additional hour at 37°. To terminate the reaction, 5 ml. of chilled physiological saline were added to each tube. For plating, 10 ml. aliquots of suspending agar, at a temperature of 42°, were inoculated with *Bact. coli* B; 1 ml. from each tube of reacted products was added and mixed; 2 ml. aliquots were plated out, incubated overnight at 37°; and the plaques counted.

Table III provides illustrative data obtained in this way with 3 polysaccharide preparations. The phage control, without any added serum, gave a highly reproducible plaque count of 276. The serum alone reduced the plaque count to 34. Each of the polysaccharides gave plaque counts, intermediate

TABLE III.—*Effect of Polysaccharide Complexes on Antiviral Action of Human Serum*

Serum*	Polysaccharide: Source	Polysaccharide: Amount (μg.)	Phage count (plaques/plate)	Per cent reversal of serum effect
0·2 ml.	None	—	33	
		—	36	0
		—	33	(serum control)
			Av. 34	
	Bryonia root	20	229	80
		15	192	65
		10	140	44
		7·5	97	26
		5·0	67	14
	Human erythrocytes	60	235	83
		40	190	64
		30	165	54
		20	116	34
		15	80	19
	Salmonella typhi	4	254	91
		3	221	77
		2	155	50
		1·5	111	32
		1·0	60	11
None	None	—	277	
		—	273	"100"
		—	278	(phage control†)
			Av. 276	

* Serum, with or without added polysaccharide, was incubated at 37° for 1 hr. Virus (1×10^4 particles) was then added. After a second incubation for 1 hr. at 37°, the reaction mixture was plated out on *Bact. coli* B.

† The plaque count given by the phage control is the value obtained on complete annulment of the serum effect, hence this level is the equivalent of 100 per cent reversal.

between these two fixed values, which were a function of the dose of polysaccharide. This annulment of the antiviral action of serum was expressed as " per cent reversal of serum effect " in the following fashion. In the case of the serum control, the per cent reversal was zero. Inasmuch as the phage control contained no serum it represented the effect that would have been obtained if the antiviral effect of serum had been entirely annulled ; hence this situation was assigned the value of 100 per cent " reversal " of serum effect. For conversion of the plaque counts to per cent effect by polysaccharide, these zero and 100 per cent values

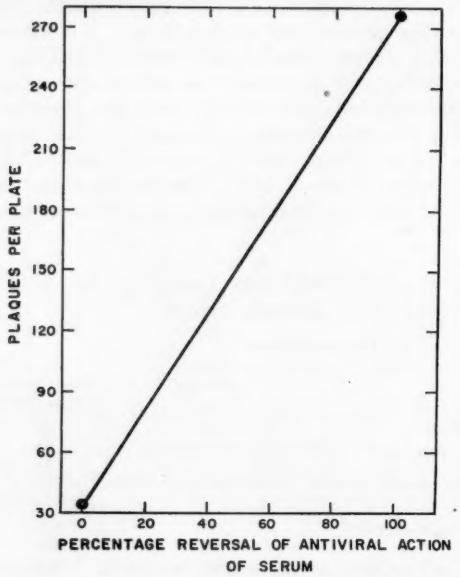

FIG. 4.—Plot for conversion of plaque counts to per cent inhibition by polysaccharide.

were plotted against their respective plaque counts, and a straight line drawn between the 2 points (Fig. 4). The per cent reversal of serum effect for each test situation was then read directly from this plot of plaque count against per cent reversal.

These values were in turn converted to probits with the use of a standard percentage response-probit table (Fisher and Yates, 1953) and plotted on probit-log paper as probits of reversal of serum action against log of the polysaccharide dose (Fig. 5). For comparing the potency of polysaccharide preparations the dose, in micrograms, which abrogates 50 per cent (probit 5) of the antiviral action of the reference serum was employed.

With the aforementioned procedure, *Bryonia* polysaccharide was assayed daily over a period of several months ; at times other polysaccharide preparations were also examined in this way. The dose-response data obtained were consistent in that more than 90 per cent of the values agreed within ± 10 per cent.

Screening of naturally occurring polysaccharides

Many polysaccharide complexes derived from Gram-negative bacteria, plants and mammalian tissues have been measured with this procedure. The dose-response data on all the preparations studied are too voluminous to be reported in their entirety. However, some of these data are given in Table IV in condensed form as representative of the results obtained on approximately 80 polysaccharides. Many of the products derived from bacteria (10 of 15) interacted with properdin without inactivating complement, whereas the majority of the tissue polysac-

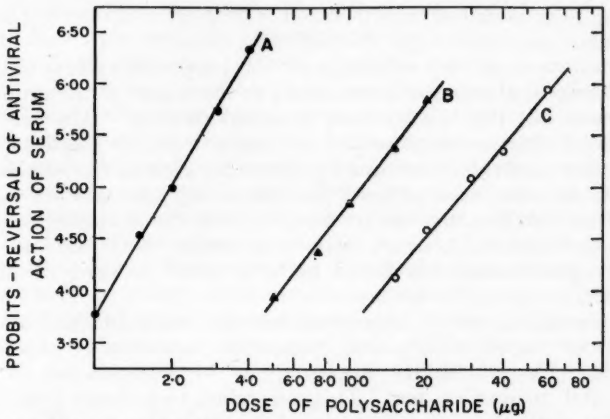

FIG. 5.—Inhibition by polysaccharide of the antiviral action of human serum. Influence of quantity of polysaccharide. A = *Salm. typhi* polysaccharide, B = *Bryonia* polysaccharide, C = human erythrocyte polysaccharide.

TABLE IV.—*Inactivation of Properdin: Relative Potency of Polysaccharide Preparations*

Category	Number of organs (or species)	Grouping of the preparations in accordance with the amount required to inactivate 0·25 units of properdin				
		0·1–1 μg.	1–10 μg.	10–13 μg.	30–100 μg.	100–300 μg.
Mammals—						
Mouse	7	—	3	—	4	—
Rabbit	2	—	2	—	—	—
Hog	1	—	1	—	—	—
Beef	1	—	1	—	—	—
Man	5	1	1	1	2	—
Plants—						
Bryonia	1	—	1	—	—	—
Yucca	1	—	—	—	1	—
Eryngium	1	—	1	—	—	—
Bacteria—						
Salmonella	4	—	1	—	—	3
Shigella	2	—	1	—	1	—
Mycobacterium	4	—	—	2	1	1
Pasteurella	2	—	1	1	—	—
Chromobacterium	1	—	—	1	—	—
Bacterium	2	—	1	1	—	—

charides (13 of 16) inactivated complement together with properdin. For brevity, the potency of these polysaccharides is tabulated in terms of the quantity, in micrograms, required for 50 per cent inactivation of 0·5 units of properdin. The majority of these products gave values in a dose range of less than 30 μg.; more of the mammalian polysaccharides than of the bacterial polysaccharides were active at low dose levels. Since there are many factors which may affect the data obtained in tests carried out at different times, we have found it useful to routinely include the same reference polysaccharide preparation when measuring the activity of varied preparations.

DISCUSSION

The neutralization of T2r$^+$ coliphage by the properdin system (Barlow *et al.*, 1958*a*) and the application of this procedure to the assay of properdin in human serum (Barlow *et al.*, 1958*b*) have been reported recently. The sensitivity and reproducibility of this procedure stand in contrast to the limited quantitation provided by other methods for measuring properdin such as the zymosan method. This reaction has now been utilized for comparing the potency of naturally occurring polysaccharides in their interaction with the properdin system. The reduction in the antiviral potency of human serum consequent to interaction with added polysaccharide was found to be a useful measure of this polysaccharide activity.

In the course of this study it became evident that individual sera of comparable antiviral activity varied widely with respect to reduction of this activity by polysaccharide. The variability appeared to be independent of properdin, complement, and magnesium ion. This was taken to indicate that, in addition to these factors, still others may be required for the interaction of properdin with polysaccharide. This situation is consistent with Pillemer's earlier observation that only 20 per cent of normal human sera are suitable for making RP (Pillemer, 1956), *i.e.*, that only a small proportion of human sera provided all the factors necessary for the complexing of zymosan with properdin. In the present study it was found that this variability was eliminated by pooling a large number of individual specimens. While this study was in progress, we learned from the late Dr. Pillemer (personal communication) that evidence was being obtained that 2 additional factors, considered to be clearly separable from the recognized components of complement, appeared to be required for the complexing of properdin with polysaccharide.

In the course of the present work we observed that many human sera did not display maximal antiviral activity unless they had been incubated prior to test; the magnitude of this effect was found to vary considerably in different sera. Accordingly, in the assay of properdin as proposed by Barlow *et al.* (1958*b*), omission of prior incubation may have an important effect on the results. However, in the polysaccharide tests here described, the incubation of polysaccharide with serum provided the effect of such " pre-incubation ".

Study of the biological properties of endotoxic polysaccharides has been, until now, restricted mainly to the effects they produce in animals. While valuable information has been obtained in this way, it is now appreciated that variability in animal response is so great that these reactions are not well suited for quantitative comparisons of different endotoxins. Among the considerations which prompted the present work was the need for a system of studying such

agents *in vitro*. In the interaction of these polysaccharide complexes with fresh serum *in vitro*, as revealed by the coliphage indicator system, not only do microgram quantities suffice, but the magnitude of the effect is critically dependent on dose.

Quantitative data on the properdin system itself are, to an increasing extent, being provided from various laboratories. The work herein described indicates the feasibility of obtaining comparable measurement of the potency of the materials which interact with this system. Although our investigation was focused on the activity of polysaccharides selected for their endotoxic properties, the usefulness of the method employed is not restricted to this type of material but is applicable to that wide category of polysaccharide complexes, endotoxic or not, which are capable of interacting with the properdin system.

In addition to making possible the quantitation of any single properdin-reactive substance, after isolation, it is conceivable that this method may be found useful for detecting or measuring such complexes in biological materials. For example, such polysaccharides might be detectable even in serum, inasmuch as they are relatively heat stable whereas the phage neutralizing activity is heat labile. Conventional immunological techniques, such as we have previously applied to the measurement of such materials *in vitro* (Landy, Trapani and Clark, 1955), are specific ; hence their reactivity is limited to individual polysaccharides. On the other hand, the present method possesses the advantage of being applicable to a whole category of these materials inasmuch as immunological specificity is not involved.

SUMMARY

The inactivation of coliphage T2r$^+$ by the properdin system in human serum may be counteracted by microgram levels of those polysaccharides which elicit endotoxic reactions in the mammalian host. Factors influencing this interaction of polysaccharide and properdin *in vitro* were examined. Under appropriate conditions of test, the antiviral action of properdin was annulled by polysaccharide as a linear function of dose. The activity of a large proportion of the polysaccharide complexes (endotoxins) derived from Gram-negative bacteria, higher plants, and mammalian tissues, was such that it could be readily measured with this procedure in a range of 1 to 30 μg.

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MUCOPROTEINS OF OX TRACHEAL CARTILAGE

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PREVIOUS investigations (Glegg, Eidinger and Leblond, 1954*a*) have revealed the presence of two alkali-soluble fractions in mammalian connective tissues, *viz.* lung, tendon, ligamentum nuchae, tracheal cartilage and bone matrix. The first fraction consisted of the acidic mucopolysaccharides, probably including the chondroitin sulphates, hyaluronic acid and keratosulphate. The second fraction contained no hexuronic acids but had a high proportion of aldoses which were identified by paper chromatography. All tissues showed the presence of galactose, mannose and fucose in the second fraction and in some, glucose also was identified. Using a periodic acid-Schiff (PAS) spot-test, the second fraction showed a strongly positive reaction, while the first fraction was non-reactive. No attempt was made to characterize the two fractions according to homogeneity, purity or chemical composition.

The present work is concerned with the electrophoretic separation and the chemical characterization of mucosubstances of ox tracheal cartilage.

EXPERIMENTAL METHODS AND RESULTS

Extraction procedure.—The fresh ox tracheal tissue (1600 g.) was minced, and extracted with 2000 ml. of 2 per cent potassium hydroxide at 4°, with intermittent shaking, for 3 days. The mixture was filtered through gauze and the liquid was centrifuged at 2500 r.p.m. This procedure was repeated twice and the combined centrifuged extracts were brought to pH 4 with glacial acetic acid. A precipitate (I) separated immediately, and, after standing for several hours at 4°, it was collected by centrifugation. Addition of 2 volumes of ethanol containing 1 per cent potassium acetate and glacial acetic acid (1 per cent) to the above supernatant gave a second precipitate (II) which, after standing for 24 hr. at 4°, was collected by centrifugation. Ethanol was added to the supernatant to give a final concentration of 85 per cent and a further precipitate (III) was collected after 24 hr. at 4°. Fraction I was washed with slightly acidified water (pH 5) and all 3 fractions washed and dried with absolute ethanol and ether.

Analysis of carbohydrate constituents.—Hydrolysis of samples from each fraction was carried out using a cationic ion-exchange resin (Zeo-Karb 225) in water at 100° for 48 hr., after the method of Glegg, Eidinger and Leblond (1954*b*). Aldoses in the hydrolysate were identified by paper chromatography on Whatman No. 1 paper using a water-saturated mixture of butanol/pyridine (4 : 1) as solvent (Chargaff, Levine and Green, 1948). Each chromatogram was run 3 times successively with ascending elution (Jeanes, Wise and Dimmler, 1951) before spraying with aniline hydrogen oxalate reagent (Horrocks and Manning, 1949). Amino-sugars retained by the Zeo-Karb resin used in the hydrolysis, were eluted with 0·5 N-HCl; these eluates were evaporated to dryness over sodium hydroxide *in vacuo*, and amino-sugars identified by the method of Stoffyn and Jeanloz (1954).

Analyses for N, S, and ash were carried out by Drs. Weiler and Strauss, Oxford. The results are shown in Table I.

Paper electrophoresis.—The 3 original fractions were examined by paper electrophoresis using Whatman No. 1 paper with a potential gradient of 2 v./cm. and a current of 2 mA applied for a period of 16 hr. Chondroitin sulphate and other metachromatic substances were revealed by staining the papers with 0·1 per cent toluidine blue in 85 per cent ethanol; after washing

the papers with 85 per cent ethanol, further treatment with 0·05 per cent bromophenol blue in 85 per cent ethanol showed the presence of protein components. Separate electrophoretic papers were stained with a periodic acid-Schiff technique described by Laurell (1955). Paper electrophoretic examination of the fractions, carried out in both 0·05 M-sodium barbitone buffer (pH 8·6), and 0·2 M-sodium acetate buffer (pH 5·0), gave the results shown in Fig. 1. Fraction I was almost insoluble in the acetate buffer (pH 5·0) and gave one faint metachromatic spot. The main component of fraction II in both buffers had the same mobility as chondroitin sulphate.

Preparative electrophoresis.—For analytical electrophoretic examination in the Tiselius moving boundary apparatus, fraction I was reprecipitated 5 times from 0·1 N-KOH by adding glacial acetic acid to pH 4. The reprecipitated fraction was dissolved in buffer solution and then dialysed for 48 hr. in cellophane at 4° against the same buffer. In each of 2 experiments using 0·1 M-glycine-NaOH buffer (pH 9·2) and 0·1 M-sodium barbitone-HCl buffer (pH 7·4), two components were observed ; a slow-moving (I*a*) and a fast-moving (I*b*). The proportion of these constituents was calculated from the areas under the curves of the Schlieren patterns and was of the order of 9 : 1 respectively. A preparative separation was effected using a large-scale Tiselius apparatus, the U-tube consisting of cells with a cross-section of 7·5 sq. cm. and a capacity of 200 ml.; the electrode vessels each held 5 l. of buffer. A 1 per cent solution of fraction I in 200 ml. of 0·1 M-glycine-NaOH buffer (pH 9·2) was dialysed against 12 l. of buffer at 4° for 48 hr. and then subjected to electrophoresis for 5 days at 1–2° using a current of 20 mA. The movement of the boundaries was followed by direct observation and isolation of the fast and slow components was achieved by the insertion of long, finely-drawn, Pasteur pipettes into the areas between the boundaries ; the solutions were withdrawn using gentle suction. The 2 fractions (I*a* and I*b*) were dialysed against distilled water, precipitated with ethanol, washed and dried with ethanol and ether. The slow-moving component (I*a*) was re-exanined electrophoretically after being redissolved in, and dialysed against, the above glycine buffer and was found to give a single peak with mobility unchanged. The 2 fractions, hydrolysed with 4 N-HCl for 4 hr., had the composition showed in Table II.

DISCUSSION

The analytical and electrophoretic results indicate that the major fraction (II) is composed mainly of chondroitin sulphate (cf. Meyer, Davidson, Linker and Hoffman, 1956), and is only weakly PAS positive. Fraction I shows some similarity to the " chondromucoid " obtained from bull trachea and whale nasal septum (Hisamura, 1938). Using a kaolin adsorption procedure " chondromucoid "

TABLE I.—*Composition of Alkali-soluble Fractions of Ox trachea*

Fraction	Per cent yield (from moist tissue)	* Per cent N	* Per cent S	* Per cent Ash	Per cent total carbohydrate (Anthrone reaction)†	$[\alpha]_D^{21}$ (in 0·1 N NaOH)	Monosaccharides present	P.A.S. spot test‡
I	1·7	10·01	1·35	0·43	6·36	−50·4	Fucose Mannose Galactose Glucosamine Galactosamine	+++
II	5·5	4·01	4·58	13·1	15·9	−21·7	Glucuronic acid Galactosamine	+
III	0·18	7·11	6·10	2·59	10·2	−28·8	Fucose Mannose Galactose Glucosamine Galactosamine	+++

* Samples dried at room temperature.
† Windrum, Kent and Eastoe, 1954.
‡ Hotchkiss, 1948.

TABLE II.—*Composition of Electrophoretically Separated Tracheal Constituents*

	Nitrogen* (per cent)	Amino-sugar† (per cent)	Uronic acid‡ (per cent)
I*a*	14·7	2·3	0·45
I*b*	5·63	12·9	14·0

* Chibnall, Rees and Williams, 1943.
† Johnston, Ogston and Stanier, 1951.
‡ Dische, 1947.

was separated by Hisamura into chondroitin sulphate and a mucoprotein (11·87 per cent N) containing both neutral sugar (7·33 per cent) and amino-sugar (8·21 per cent). Fraction I*a* produced by our preparative electrophoretic separation,

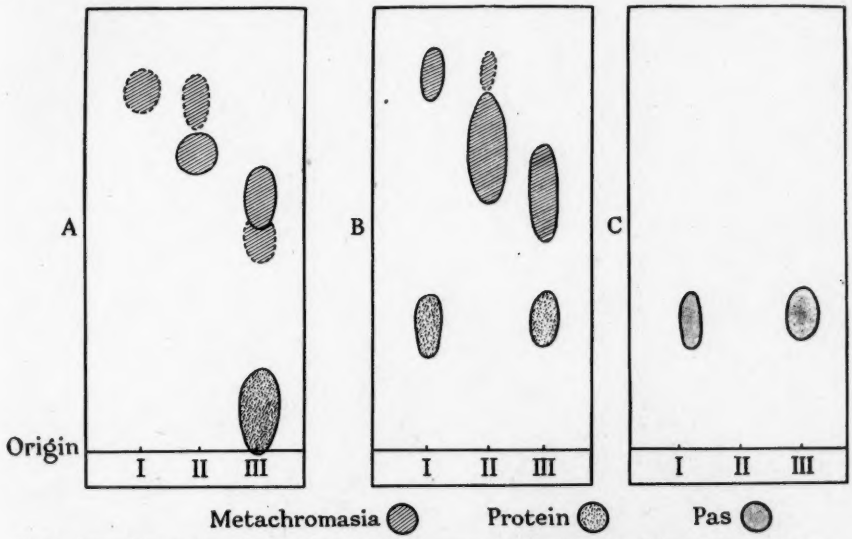

FIG.—Separation of fractions I, II and III by paper electrophoresis. Migration of anionic tracheal mucoprotein fractions on Whatman No. 1 paper after 16 hr. at potential gradient of 2 v./cm. A run in 0·2 M acetate buffer. B and C run in 0·05 M barbiturate buffer.

appears to be similar to Hisamura's mucoprotein. The composition of I*b*, however, suggests that it is a complex formed from protein and chondroitin sulphate. The occurrence of such a complex in bovine nasal and tracheal cartilage has already been reported (Shatton and Schubert, 1954; Webber and Bayley, 1956; Partridge and Davies, 1958; Malavista and Schubert, 1958) and their results indicate that I*a* closely resembles the non-collagenous protein moiety which has been found (Partridge and Davies, 1958) to be liberated from the chondroitin sulphate protein complex by alkaline hydrolysis. A strong PAS reaction was shown by both fractions I and III; paper electrophoresis (Fig. 1) showed this staining reaction to be localized in the slow-moving protein-staining components. It seems probable, therefore, that some or all of the monosaccharides demonstrated by paper chromotography in fractions I and III, are combined with these proteins. This would also apply to the electrophoretically separated fraction I*a* if it is assumed

to be identical with the slower moving component of I noticed in paper electrophoresis. Fractions I and III show similarities with regard to their PAS/protein staining fractions but differ markedly in their solubilities in water. The insolubility of I, compared to the solubility of III may be related to the degradation of the protein by the extraction procedure. Such a degradation may have occurred at one stage of the extraction procedure used by Glegg *et al.* (1954*a*), and would explain why they did not find a water-insoluble fraction ; the aldoses were found all to occur in the fraction requiring 85 per cent ethanol for precipitation. Whether both soluble and insoluble forms of the protein occur normally requires further investigation.

These methods are now being applied to the isolation of mucoproteins from bone and preliminary experiments indicate the presence of similar PAS reactive fractions.

SUMMARY

Three fractions have been obtained by alkaline extraction of tracheal cartilage. One of these (II) consists mainly of chondroitin sulphate. The other fractions (I and III) contain fucose, mannose, galactose and amino-sugars and on paper electrophoresis showed components which contained protein and were PAS positive. A preparative electrophoretic separation was carried out on fraction I, the main component of which proved to be a mucoprotein which is considered to contain neutral sugars and therefore to be responsible for the strong PAS reaction.

This work was begun on behalf of the Medical Research Council's Committee on Protection Against Ionizing Radiation. One of us (G. M. H.) was paid from a grant for assistance and expenses to Janet Vaughan from the Medical Research Council.

We are grateful to Dr. M. Tombs for his advice and assistance with the Tiselius electrophoresis apparatus.

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STUDIES ON THE MECHANISM OF SHOCK. THE QUANTITATIVE ASPECTS OF GLYCOGEN METABOLISM AFTER LIMB ISCHAEMIA IN THE RAT

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THE mechanism of the general response to injury (shock) can be studied through its associated metabolic changes. The obvious effect of injury on energy metabolism directs attention to the changes in carbohydrate metabolism. Although it is generally agreed that the glycogen concentration in the damaged tissue, liver and muscle outside the damaged area is reduced the changes in other tissues are less certain. Little attention has been paid to the causation of the changes, their relation to the other consequences of injury, the influence of nutritional status on these changes and the total amounts of glycogen involved. The present paper is mainly concerned with the quantitative aspects of this problem.

Previous work (Stoner, 1958) has shown that the survival period in ischaemic shock can be divided into two stages depending on the adequacy of tissue oxygenation. The present results show how this is reflected in the changes in carbohydrate metabolism after limb ischaemia and emphasize the importance of the adrenal medulla in bringing about these changes. They also show that glycogen formation from glucose continues in the tissues even when they are unable to store glycogen but they do not provide any evidence for an increase in the formation of glucose from non-carbohydrate sources in ischaemic shock in these conditions.

METHODS

Experiments were performed on albino rats of the Porton strain (body wt. 222 ± 17 g.) fed on MRC diet 41 or 41b (Bruce and Parkes, 1949, 1956). Both diets contain 13·6 per cent digestible protein, 48·4 per cent soluble carbohydrate and 4·5 per cent fat, the only difference being the replacement of the cod liver oil in diet 41 by a stabilized vitamin supplement. Food and water were given *ad lib* until the start of the experiment when food was removed. When the rats were fasted, food was removed 24 hr. before the experiment and coprophagy prevented. Rats were brought to the laboratory from the animal house about a week before the experiment to ensure that they were acclimatized to laboratory conditions and that their diurnal feeding rhythm was normal (Stoner, 1956).

Adrenal medullectomy was performed by the method of Evans (1936) as quoted by Ingle and Griffiths (1942). These rats were given 1 per cent NaCl to drink for 1 week postoperatively and used after 53–67 days. The absence of medulla was confirmed histologically. Adrenalectomy was carried out under ether anaesthesia through a dorsal approach and the rats were given 1 per cent NaCl to drink afterwards. They were used after 4 days.

Bilateral hind-limb ischaemia was produced with rubber tourniquets (Rosenthal, 1943) applied during a 3 min. period of ether anaesthesia. Controls had a similar anaesthetic. The environmental temperature was 18–22°.

The tissues were sampled under Na pentobarbitone anaesthesia (5 mg. per 100 g. body wt., intraperitoneally; Veterinary Nembutal-Abbott). For the brain, as soon as the rat was

anaesthetized it was plunged head first into liquid N_2 and the cerebrum dissected out in the frozen state. •For muscle and heart glycogen, samples of the right pectoralis major, both ventricles and right gastrocnemius were removed in that order and frozen with liquid N_2 as quickly as possible. For liver glycogen, samples from the pars centralis (Gershbein and Elias, 1954) were put into tared tubes containing 40 per cent KOH for "total glycogen" or weighed and ground up with 10 per cent trichloroacetic acid (redistilled, A.R.) in a Potter-Elvehjm all-glass homogenizer for "acid-soluble glycogen" (Bloom, Lewis, Shumpert and Shen, 1951). Blood samples were obtained from the thorax with a Pasteur pipette after division of the great vessels and heparin used as an anti-coagulant. Haematocrit values were obtained with Wintrobe tubes and corrected for trapped plasma (Chaplin and Mollison, 1952).

Blood sugar was estimated by the method of Haslewood and Strookman (King, 1946). Total glycogen was determined by the method of Good, Kramer and Somogyi (1933), the glucose liberated by hydrolysis being measured by Nelson's method (1944). For brain the cerebrosides were removed from the ethanol precipitate as described by Kerr (1936). Acid soluble liver glycogen was determined by the method of Bloom *et al.* (1951).

Thin slices of liver were fixed in Rossman's fluid for the histochemical demonstration of glycogen by the periodic acid-Schiff reaction with saliva control.

Uniformly ^{14}C-labelled glucose (Radiochemical Centre, Amersham) was injected into a tail vein in 0·9 per cent NaCl. After 2 hr. the rat was anaesthetized with Na pentobarbitone and the liver, hind-limb muscle and as much carcase muscle as possible rapidly excised and frozen in liquid N_2. Pure samples of the glycogen in these tissues were prepared according to the methods of Somogyi (1934) or Stetten, Katzen and Stetten (1954). The CO_2 obtained by combustion of the glycogen according to the method of Lindenbaum, Schubert and Armstrong (1948) was absorbed in NaOH. $BaCO_3$ was then precipitated with $BaCl_2$ and filtered on to sintered discs. The radioactivity of the $BaCO_3$ was determined at infinite thickness with an end-window counter (EHM 2S; G.E.C.) attached to conventional scaling apparatus. A methylmethacrylate ^{14}C standard was used for comparison.

Results are expressed where possible as the mean $\pm$ standard deviation (S.D.), and statistical comparisons of the means made according to Student's t test (Fisher, 1934).

RESULTS

General Effects of Limb Ischaemia

The mortality rate and survival time after a 4 hr. period of bilateral hind-limb ischaemia at an air temperature of 18–22° are 85 per cent and 13·28 $\pm$ 0·90 hr. (Threlfall and Stoner, 1957). Fasting increases the mortality rate to 100 per cent and shortens the survival time (Threlfall and Stoner, 1954). The poorer condition of the fasted rats was evident within 3 hr. of removing the tourniquets. Adrenal medullectomy also raised the mortality rate to 100 per cent and shortened the survival time to 5·79 $\pm$ 4·72 hr. After total adrenalectomy the survival time was decreased still further to 1·86 $\pm$ 0·67 hr.

Fed normal rats

Blood sugar (Fig. 1).—Hyperglycaemia appeared during the ischaemic period and increased to a maximum about 3 hr. after the tourniquets had been removed. The level then fell progressively, reaching hypoglycaemic levels in fatal cases.

Liver glycogen (Fig. 2 and 3).—Part of the normal diurnal variation (Higgins, Berkson and Flock, 1933; Deuel, Butts, Hallman, Murray and Blunden, 1938) is shown by the control curve in Fig. 2. Normally the rat would begin to feed again about 6.0 p.m. but in the absence of food the fall continued until 4.0 a.m. The diurnal cycle is not completely abolished during the first 24 hr. of a fast (Higgins *et al.*, 1932) so that the 10.0 a.m. level in the 24 hr. fasted rats (Table I) was higher than that at 4.0 a.m.

At the end of the 4 hr. period of limb ischaemia the total liver glycogen was higher than would be expected from the controls. After removal of the tourniquets it was nearly always below that in the controls and within a few hours reached very low levels which persisted until death. Similar changes occurred in the acid-soluble glycogen level (Fig. 3). Histochemically the periportal cells were depleted of glycogen before the central cells in the same way as in the controls.

Muscle glycogen (Fig. 4).—The muscle glycogen level in the fed rat fell gradually to the level shown in Table I after a 24 hr. fast. With hind-limb ischaemia the

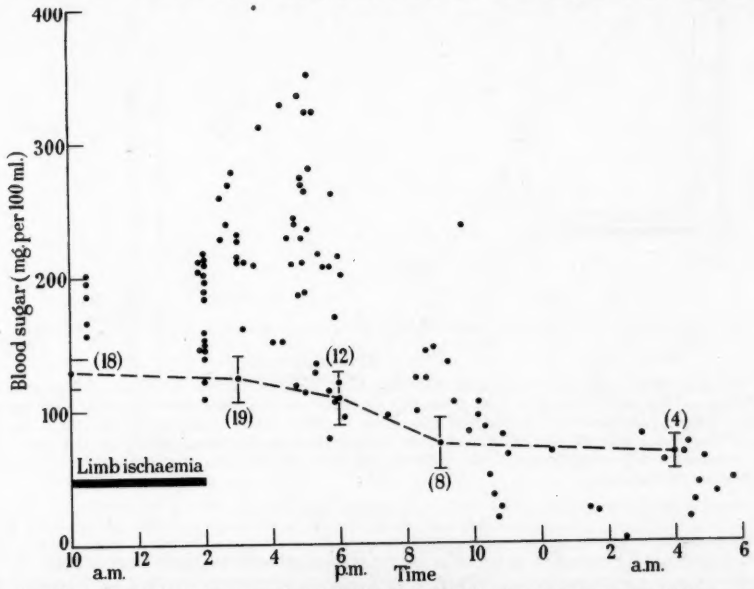

FIG. 1.—The effect of a 4 hr. period of bilateral hind-limb ischaemia between 10.0 a.m. and 2.0 p.m. on the blood sugar level of the fed rat, each point indicates the level in individual rats. The mean blood sugar level in fed control rats given a short ether anaesthesia at 10.0 a.m. is shown by the curve ●– – –● where each point indicates the mean of results in the number of rats shown in parentheses and the vertical line through the point represents the standard deviation of the mean.

glycogen contents of the pectoralis and gastrocnemius muscles fell similarly during the first 2 hr. of the experiment but during the next 2 hr. the fall was greater in the ischaemic muscle. If the tourniquets were kept on, this fall in the gastrocnemius continued so that after 6 hr. ischaemia its glycogen level was 108 $\pm$ 96 mg. glucose per 100 g. wet wt.

When the tourniquets were removed after 4 hr. the glycogen level in the pectoralis major continued to fall at about the same rate as before. The level in the gastrocnemius also continued to fall at first but after a few hours it rose and this higher level persisted. While the level in the gastrocnemius was below that in the pectoralis major when the tourniquets were removed, a few hours later it was consistently higher. This is shown in the later part of Fig. 4 where the pairs of points at any one time refer to the same rat. Examination of these extracts after

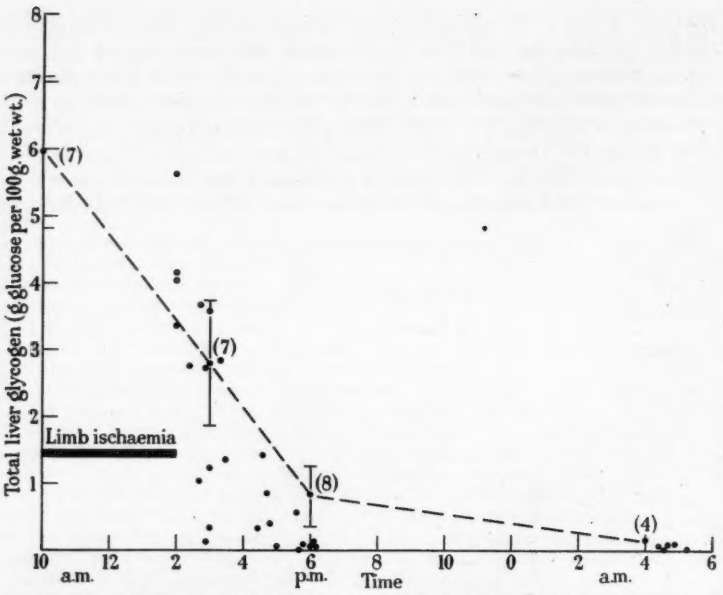

FIG. 2.—The effect of a 4 hr. period of bilateral hind-limb ischaemia between 10.0 a.m. and 2.0 p.m. on the total liver glycogen of the fed rat, each point indicates the level in individual rats. The mean level in fed control rats given a short ether anaesthesia at 10.0 a.m. is shown by the curve ●– – –● where each point indicates the mean results in the number of rats shown in parentheses and the vertical line through the point represents the standard deviation of the mean.

TABLE I.—*The Effect of a 4 hr. Period of Bilateral Hind-limb Ischaemia Between 10.0 a.m. and 2.0 p.m. on the Blood Sugar and Tissue Glycogen Levels (Mean ± S.D.) in Fasted Normal Rats*

(Number of observations shown in parentheses)

Group	Time	Blood sugar (mg. per 100 ml.)	Glycogen (mg. glucose per 100 g. wet wt.)			
			Ventricles	Liver	Pectoralis major	Gastrocnemius
Control	9.30 a.m.	76±21 (10)	504±55 (3)	251±114 (5)	337±90 (4)	311±110 (5)
,,	2.0 p.m.	69±14 (11)	499±24 (4)	84±65 (6)	249±90 (5)	389±95 (6)
,,	5.0 p.m.	55±14 (8)	447±55 (4)	84±62 (4)	250±123 (6)	365±69 (6)
Tourniquet	2.0 p.m.	86±25 (12)	507±83 (5)	273±146* (6)	120±52* (4)	36±37‡ (5)
,,	5.0 p.m.	90±29† (8)	450±44 (4)	20±13 (4)	65±36† (6)	132±113† (6)

* Significantly different from appropriate control mean at $P < 0{\cdot}05$.
† ,, ,, ,, ,, ,, ,, ,, $P < 0{\cdot}01$.
‡ ,, ,, ,, ,, ,, ,, ,, $P < 0{\cdot}001$.

acid hydrolysis and after neutralization by passing them down a column of Amberlite IR-4B(OH) showed that the reducing substance was yeast fermentable. Results obtained when glucose was added during the initial preparation of the muscle extracts excluded possible contamination by hyperglycaemic blood. This increase in the glycogen content of the post-ischaemic muscle was prevented by lengthening the period of ischaemia to 6 hr.

No attempt was made to divide the muscle glycogen into an acid-soluble and residual fraction since, in agreement with Carroll, Longley and Roe (1956), we

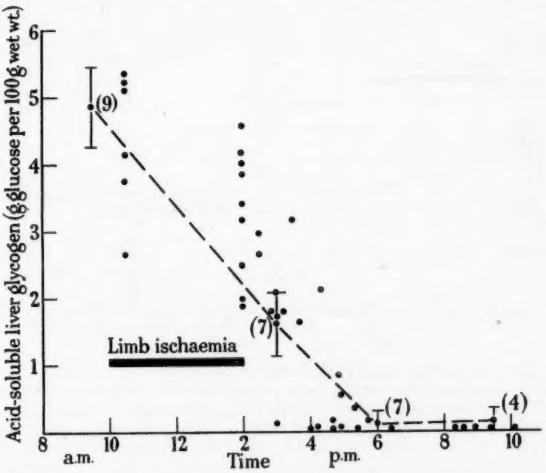

FIG. 3.—The effect of a 4 hr. period of bilateral hind-limb ischaemia between 10.0 a.m. and 2.0 p.m. on the acid soluble liver glycogen of the fed rat, each point indicates the level in individual rats. The mean level in fed control rats given a short ether anaesthesia at 10.0 a.m. is shown by the curve ●– – –● where each point indicates the mean of results in the number of rats shown in parentheses and the vertical line through the point represents the standard deviation of the mean.

have found that with efficient homogenization practically all the muscle glycogen is extractable with trichloroacetic acid.

Brain glycogen (Fig. 5).—The total glycogen content of the control brains showed little diurnal variation and the level in the experimental rats only fell when they were moribund.

Ventricular glycogen (Fig. 6).—The total ventricular glycogen level in the fed control rat rose steadily after removal of food at the beginning of the experiment. At the end of the limb ischaemia the level was considerably above that in the controls and this increase continued for about 2 hr. The level then returned to normal limits where it remained for most of the survival period. Terminally it was reduced.

Fasted normal rats

Fig. 1–6 show that an assessment of the major glycogen shifts could be made by comparing the levels at the removal of the tourniquets and 3 hr. later with the

control levels at 9.30 a.m., 2.0 and 5.0 p.m. The results in rats fasted for 24 hr. before the experiment are given in Table I.

Fasting greatly reduced the hyperglycaemic response to limb ischaemia and prevented the rise in the ventricular glycogen level. Liver glycogen again rose during limb ischaemia and then fell to a very low level, about the same as in the fed rats. The percentage reduction in the glycogen content of the pectoralis major was greater than in the fed rats. Glycogen deposition occurred in the gastrocnemius after removal of the tourniquets in all but 1 of the 6 rats.

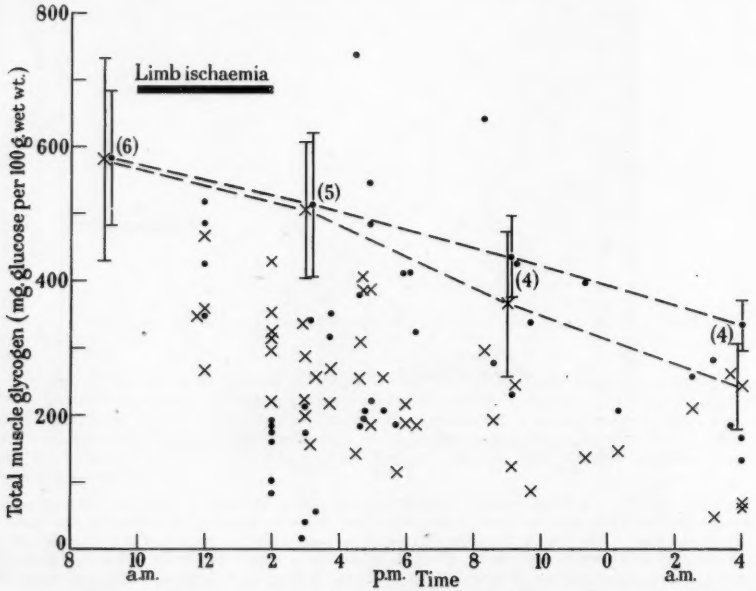

FIG. 4.—The effect of a 4 hr. period of bilateral hind-limb ischaemia between 10.0 a.m. and 2.0 p.m. on the total muscle glycogen in the pectoralis major (×) and gastrocnemius (●) of the fed rat, each point indicates the levels in individual rats. The mean levels in fed control rats given a short ether anaesthesia at 10.0 a.m. are shown by the curves, ×– – –× for the pectoralis major, ●– – –● for the gastrocnemius where each point indicates the mean of results in the number of rats shown in parentheses and the vertical lines through the points represent the standard deviations of the means.

Fed adrenal medullectomized rats

These results are shown in Table II. Medullectomy prevented the hyperglycaemia and 3 hr. after removal of the tourniquets the blood sugar level was significantly below that in the controls. The early onset of hypoglycaemia was in keeping with their poor clinical state. Medullectomy did not prevent the increase in ventricular glycogen. In some rats, not included in Table II, this rise continued for about 2 hr. after the period of limb ischaemia. The level then fell gradually, the final concentration being similar to that in injured normal rats at death. The liver glycogen level was unchanged during the limb ischaemia but fell afterwards at about the same rate as in the fed normal rats injured in this way. The glycogen

content of the undamaged muscle remained unaltered up to the time of death. The level in the ischaemic muscle fell as in the normal rats but there was no significant storage of glycogen in the post-ischaemic period and terminally the level fell again.

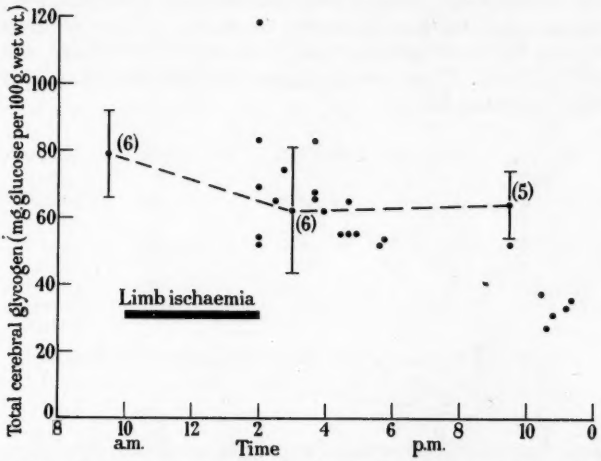

FIG. 5.—The effect of a 4 hr. period of bilateral hind-limb ischaemia between 10.0 a.m. and 2.0 p.m. on the brain glycogen of the fed rat, each point indicates the level in individual rats. The mean level in fed control rats given a short ether anaesthesia at 10.0 a.m. is shown by the curve ●– – –● where each point indicates the mean of results in the number of rats shown in parantheses and the vertical line through the point represents the standard deviation of the mean.

TABLE II.—*The Effect of a 4 hr. Period of Bilateral Hind-limb Ischaemia Between 10.0 a.m. and 2.0 p.m. on the Blood Sugar and Tissue Glycogen Levels (Mean ± S.D.) in Fed Medullectomized Rats*

(Number of observations shown in parentheses)

Group	Time	Blood sugar (mg. per 100 ml.)	Glycogen (mg. glucose per 100 g. wet wt.)			
			Ventricles	Liver	Pectoralis major	Gastrocnemius
Control	9.30 a.m.	114±8 (8)	384±72 (5)	6740±711 (3)	659±208 (5)	660±159 (5)
,,	2.0 p.m.	118±10 (7)	416±72 (3)	3895±1386 (4)	497±114 (5)	526±140 (5)
,,	5.0 p.m.	117±19 (5)	381±19 (3)	1540±544 (3)	491±130 (5)	414±157 (5)
Tourniquet	2.0 p.m.	130±30 (8)	589±21† (4)	4278±1096 (5)	520±76 (5)	159±36‡ (5)
,,	5.0 p.m.	61±41* (8)	486±102 (4)	275±294* (4)	659±174 (5)	196±143* (5)
,,	At death	33±9‡ (5)	271±60* (3)	79±23† (4)	529±121 (3)	62±43‡ (3)

* Significantly different from the control mean at $P < 0{\cdot}05$.
† ,, ,, ,, ,, ,, ,, ,, $P < 0{\cdot}01$.
‡ ,, ,, ,, ,, ,, ,, ,, $P < 0{\cdot}001$.

Fed adrenalectomized rats

In these rats (Table III) not only was hyperglycaemia prevented but when the tourniquets were removed the blood sugar level fell rapidly. The control glycogen levels were generally lower and more variable than in the other groups. In the injured rats the ventricular glycogen level was reduced by the end of the period of limb ischaemia and fell further when the tourniquets were removed. The liver glycogen level also fell more quickly than in the controls. There was no recovery in the glycogen content of the post-ischaemic muscle and that of the uninjured muscle showed a terminal fall.

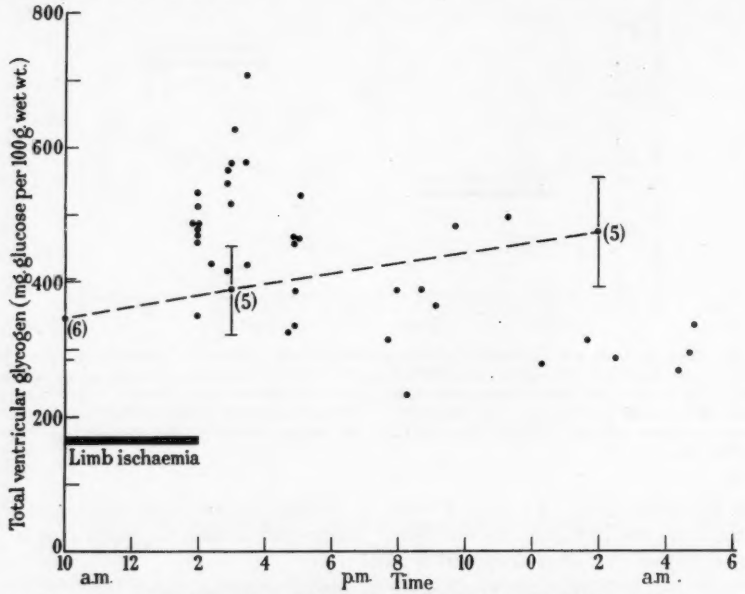

FIG. 6.—The effect of a 4 hr. period of bilateral hind-limb ischaemia between 10.0 a.m. and 2.0 p.m. on the total ventricular glycogen of the fed rat, each point indicates the level in individual rats. The mean level in fed control rats given a short ether anaesthesia at 10.0 a.m. is shown by the curve ●– – –● where each point indicates the mean of results in the number of rats shown in parentheses and the vertical line through the point represents the standard deviation of the mean.

Effect of Limb Ischaemia on Glycogen Storage and Turnover

The ability of the rat, under certain conditions, to store glycogen in the myocardium and in the muscles of the damaged limb after removal of the tourniquets has already been described.

When rats were given large amounts of glucose either by mouth (1·0 ml. 25 per cent aqueous soln. per 100 g. body wt.) or intravenously (0·5 ml. 6 per cent aqueous soln. per 100 g. body wt.) after a 4 hr. period of bilateral hind-limb ischaemia no effect was seen on the muscle glycogen level but the total liver glycogen concentration sometimes increased. This only occurred when the glucose was given shortly after the tourniquets had been removed. As the blood sugar

TABLE III.—*The Effect of a 4 hr. Period of Bilateral Hind-limb Ischaemia Between 10.0 a.m. and 2.0 p.m. on the Blood Sugar and Tissue Glycogen Levels (Mean ± S.D.) in Fed Adrenalectomized Rats*

(Number of observations shown in parentheses)

Group	Time	Blood sugar (mg. per 100 ml.)	Glycogen (mg. glucose per 100 g. wet wt.)			
			Ventricles	Liver	Pectoralis major	Gastrocnemius
Control	9.30 a.m.	119±11 (8)	308±50 (5)	2628±1400 (8)	346±128 (6)	374±112 (6)
,,	2.0 p.m.	101±21 (11)	252±54 (5)	537±271 (12)	312±87 (6)	357±55 (7)
,,	5.0 p.m.	63±18 (5)	344±49 (4)	114±88 (5)	363±37 (5)	359±40 (5)
Tourniquet	2.0 p.m.	86±36 (11)	179±18* (6)	170±136‡ (10)	338±68 (7)	45±24‡ (7)
,,	At death (approx. 4.0 pm.)	20±6‡ (8)	115±56‡ (5)	65±43 (7)	268±35† (5)	38±12‡ (5)

* Significantly different from the control mean at $P < 0{\cdot}05$.
† ,, ,, ,, ,, ,, ,, ,, $P < 0{\cdot}01$.
‡ ,, ,, ,, ,, ,, ,, ,, $P < 0{\cdot}001$.

levels in the rats given oral doses of glucose were within the range for the untreated rats it is questionable how much of the glucose was absorbed.

The frequent inability of the injured rat to store glycogen in the skeletal muscle and liver does not mean that it was unable to synthesize it. The incorporation of ^{14}C-glucose into the glycogen of these tissues continued after limb ischaemia (Table IV) and the few available results suggest that this was decreased in the

TABLE IV.—*The Radioactivity (μc. per g. glucose) of the Glycogen Prepared from the Tissues of Rats 2 hr. After the Intravenous Injection of 20 μc. ^{14}C-glucose per 100 g. body wt.*

The rats were fed overnight and at 10.0 a.m. bilateral hind-limb tourniquets were applied under ether anaesthesia and the controls given ether. The tourniquets were removed at 2.0 p.m. and the ^{14}C-glucose injected in both groups at 2.15 p.m.

	Glycogen radioactivity (μc. per g. glucose)		
	Muscle (exc. hind-limb)	Hind-limb muscle	Liver
Control rat	0·932	0·475	0·146
,, ,,	3·010	1·655	0·440
,, ,, *	1·372†	—	0·188
Experimental rat	0·418	2·850	0·230
,, ,,	—	11·100	0·475
,, ,, *	0·381	14·800	0·460

* Glycogen prepared by method of Stetten *et al.* (1956), others prepared by method of Somogyi (1934).
† Including hind-limb muscle.

undamaged muscle and increased in the liver and injured muscle during the early part of the response. The last finding was in keeping with the increase in the glycogen content of the damaged muscle at that time.

Effect of Limb Ischaemia on " Total Body Glucose "

So far results have been expressed per unit tissue weight. They can also be expressed per 100 g. body wt. giving a value for the total amount of glucose in the body either free or combined as glycogen. This value will be called the " total body glucose ". The method of calculation is as follows :

Values of 4·15, 0·55 and 0·289 have been taken as the weights of the liver, brain and heart as percentages of the body wt. (Caster, Poncelet, Simon and Armstrong, 1956). A negligible error will be introduced by equating the glycogen level in the ventricles with that in the whole heart (Davies, Francis and Stoner, 1947). The damaged muscle in the hind-limbs was, by dissection, 8 per cent of the body wt., leaving 37·5 as the percentage for the undamaged muscle (Caster *et al.*, 1956). The justification for using the glycogen content of one muscle as a measure of that in the whole musculature has been discussed by Winternitz, Dintzis and Long (1957). In freshly drawn rat blood, the blood glucose is almost entirely confined to the plasma (unpublished observations) enabling the plasma concentration to be calculated from knowledge of the haematocrit (Table V). Steele, Wall, de Bodo and Altszuler (1956) have shown that glucose is distributed through the extracellular space of the body at its concentration in the plasma. The total extracellular space has been taken as 20 per cent of the body wt. (Manery, 1954) and it is considered to be unaltered by the injury (Green and Stoner, 1950 ; Rosenthal and Millican, 1954).

TABLE V.—*The Effect of a 4 hr. Period of Bilateral Hind-limb Ischaemia on the Haematocrit of the Rat*

	Average haematocrit (corrected)				
			Time (hr.) after ischaemia		
Group	Before ischaemia	At end of ischaemia	1	3	14
Intact	43	43	54	60	57
Medullectomized	42	41	—	70	—
Adrenalectomized	41	48	57*	—	—

* At death, approx. 2 hr. after ischaemia.

The results of these calculations are shown in Tables VI–IX. These total values are of the same order as those obtained for the " total body carbohydrate " (Threlfall and Stoner, 1954) indicating that no major source of glycogen has been omitted. The possible variability of these estimates of " total body glucose " is difficult to assess. The figures in parentheses below the total values in the table show the percentage increase which would have occurred if the mean glucose levels in the compartments had all been raised one S.D.

In intact rats subjected to limb ischaemia the loss of " total body glucose " during the ischaemic period was greater than in the controls during this period of the day. This was followed by a phase of normal consumption which was succeeded by a terminal phase of accelerated consumption. Medullectomy and adrenalectomy prevented the initial loss but the terminal phase commenced sooner.

TABLE VI.—*The Glucose Content of the Extracellular Space and the Glucose Present in the Organs as Glycogen Expressed as mg. glucose per* 100 *g. body wt. in Control Rats Fed up to the Time of the First Observations and in Similar Rats Subjected to Bilateral Hind-limb Ischaemia Between* 10.0 *a.m. and* 2.0 *p.m.*

Method of calculation and significance of figures in parentheses given in the text. The values used to calculate the amounts in the controls at 2.0 and 5.0 p.m. obtained by interpolation from the curves in Fig. 1, 2, 4, 5 and 6.

Time	9.30 a.m.	2.0 p.m.	3.0 p.m.	5.0 p.m.	6.0 p.m.	9.0 p.m.	4.0 a.m.
Controls :							
Extracellular space	45·2	44·0	43·6	40·6	38·2	26·0	23·2
Liver	246.5	143·0	116·2	62·3	33·6	24·9	5·6
Brain	0·4	0·4	0·3	0·3	0·4	0·4	0·4
Heart	1·0	1·1	1·1	1·2	1·2	1·2	1·4
Muscle (exc. hind-limb)	218·3	194·0	189·4	172·2	163·1	136·5	91·1
Hind-limb muscle	46·6	42·1	41·0	39·2	38·0	34·9	26·7
Total	558·0	424·6	391·6	315·8	274·5	183·9	148·4
	(20·9)	—	(21·9)	—	—	(48·2)	—
Experimental :							
Extracellular space	—	60·8	—	117·0	—	55·8	20·6
Liver	—	178.0	—	16·1	—	2·0	0·2
Brain	—	0·4	—	0·3	—	0·2	0·2
Heart	—	1·4	—	1·2	—	1·1	0·9
Muscle (exc. hind-limb)	—	120·5	—	98·8	—	70·5	48·8
Hind-limb muscle	—	12·0	—	26·7	—	30·5	16·4
Total	—	373·1	—	260·1	—	160·1	87·1

TABLE VII.—*The Glucose Content of the Extracellular Space and the Glucose Present in the Organs as Glycogen Expressed as mg. glucose per* 100 *g. body wt. in Control Rats After* 24 *hr. Fast and in Similar Rats Subjected to Bilateral Hind-limb Ischaemia, Between* 10.0 *a.m. and* 2.0 *p.m.*

Method of calculation and significance of figures in parentheses given in the text.

Time	9.30 a.m.	2.0 p.m.	5.0 p.m.
Controls :			
Extracellular space	26·6	24·2	19·3
Liver	10·4	3·5	3·5
Heart	1·5	1·4	1·3
Muscle (exc. hind-limb)	126·4	93·4	93·8
Hind-limb muscle	24·9	31·1	29·2
Total	189·8	153·6	147·1
	(28·8)	(31·9)	(40·3)
Experimental :			
Extracellular space	—	30·4	45·0
Liver	—	11·3	1·0
Heart	—	1·5	1·3
Muscle (exc. hind-limb)	—	45·0	24·4
Hind-limb muscle	—	5·2	10·6
Total	—	93·4	82·3
	—	(37·4)	(45·5)

TABLE VIII.—*The Glucose Content of the Extracellular Space and the Glucose Present in the Organs as Glycogen Expressed as mg. glucose per* 100 *g. body wt. in Control Adrenal Medullectomized Rats Fed up to the Time of the First Observations and in Similar Rats Subjected to Bilateral Hind-limb Ischaemia Between* 10.0 *a.m. and* 2.0 *p.m.*

Method of calculation and significance of figures in parentheses given in the text.

Time	9.30 a.m.	2.0 p.m.	5.0 p.m.	
Controls :				
Extracellular space	39·3	40·7	40·3	—
Liver	279·7	161·6	63·9	—
Heart	1·1	1·2	1·1	—
Muscle (exc. hind-limb)	247·1	186·4	184·1	—
Hind-limb muscle	52·8	42·1	33·1	—
Total	620·0	432·0	322·5	—
	(19·9)	(21·7)	(27·3)	—
Experimental :				
Extracellular space	—	44·1	40·7	22·0*
Liver	—	177·5	11·4	3·3
Heart	—	1·7	1·4	0·8
Muscle (exc. hind-limb)	—	195·0	247.1	198·4
Hind-limb muscle	—	12·7	15·7	5·0
Total	—	431·0	306·3	229·5
	—	(15·9)	(41·2)	(24·3)

* At death.

TABLE IX.—*The Glucose Content of the Extracellular Space and the Glucose Present in the Organs as Glycogen Expressed as mg. glucose per* 100 *g. body wt. in Control Adrenalectomized Rats Fed up to the Time of the First Observations and in Similar Rats Subjected to Bilateral Hind-limb Ischaemia Between* 10.0 *a.m. and* 2.0 *p.m.*

Method of calculation and significance of figures in parentheses given in the text.

Time	9.30 a.m.	2.0 p.m.	5.0 p.m.
Controls :			
Extracellular space	40·3	34·2	21·4
Liver	109·1	22·3	4·7
Heart	0·9	0·7	1·0
Muscle (exc. hind-limb)	129·8	117·0	136·1
Hind-limb muscle	29·9	28·6	28·7
Total	310·0	202·8	191·5
	(37·9)	(27·4)	(33·9)
Experimental :			
Extracellular space	—	33·1	9·3*
Liver	—	7·1	2·7
Heart	—	0·5	0·3
Muscle (exc. hind-limb)	—	126·8	100·5
Hind-limb muscle	—	3·6	3·0
Total	—	171·1	115·8
		(27·4)	(16·3)

* At death, approx. 4.0 p.m.

DISCUSSION

The effects of limb ischaemia on carbohydrate metabolism can be divided into three stages.

Changes during limb ischaemia

This period is characterized by a fall in the muscle glycogen level, a rise in the levels in the liver and heart and an increase in the amount of extracellular glucose. The release of glycogen from the uninjured muscle as lactate and pyruvate would be more than sufficient to account for the increase in the glucose content of the other compartments in both fed and fasted rats (Tables VI and VII) even allowing for the cost of the conversion of the lactate and pyruvate (Meyerhof, 1920). These changes are typical of the Cori cycle in the rat (Cori and Cori, 1928) and their suppression, except for those in the ventricles, by adrenal medullectomy shows that they are due to the release of adrenaline in response to the injury. The increased loss of " total body glucose " during this period could be explained by the metabolic cost of the continued movement of lactate and pyruvate through this cycle.

The rise in ventricular glycogen, first described by Cordier and Dessaux (1954), was not prevented by medullectomy in fed rats but did not occur in intact fasted rats or in fed adrenalectomized rats. Hormonal mechanisms are probably not directly involved in this. Very little glycogen is actually formed (Table VI). The effect does not depend on hyperglycaemia and the increase may be derived from the circulating lactate and pyruvate both of which can be utilized by the heart (see Evans, 1949) although they do not appear to form glycogen in the perfused heart (Bogue, Evans and Gregory, 1937; Braun-Menendez, Chute and Gregory, 1939). After medullectomy these compounds will still be released from damaged muscle just above the tourniquets. In the fasted rat the initial glycogen level is already maximal (Adronny and Russell, 1956) and after adrenalectomy the terminal accelerated destruction has already begun when the tourniquets are removed.

After limb ischaemia I

This stage lasts about 3–6 hr. and the changes during it are the most difficult to interpret. During this period total O_2 consumption and tissue temperature are falling rapidly although the liver, brain and muscle appear to be adequately oxygenated (Stoner, 1958).

In the fed rat the blood sugar rises to its maximum and there is a continuing fall in the glycogen content of the uninjured muscle. The level in the liver fell steeply but that in the ventricles continued to rise and there was some recovery in the damaged muscle. Brain glycogen was unaltered. Previous fasting reduced the size of most of these changes but the amount of glycogen lost from the uninjured muscle remained the same (Tables VI, VII). The mechanism of some of these changes is clear.

When the tourniquets are removed the circulation is flooded with lactate and pyruvate and this could account for the further rise in ventricular glycogen (see above). The latter did not occur in the experiments of Cordier and Dessaux

(1954) because the high environmental temperature (30°) they used would precipitate the onset of the final stage (Stoner, 1958).

Medullectomy prevented the hyperglycaemia and fall in the glycogen level in the uninjured muscle so that these changes can be attributed to the further release of adrenaline. Adrenaline does not affect the cerebral glycogen level (Kerr and Ghantus, 1936) so that this level remains unchanged. The constancy of this level supports a previous conclusion (Stoner, 1958) that the brain is adequately oxygenated for most of the survival period.

The glycogen stored in the damaged muscle appears to come from the circulating glucose (Table IV) and to reflect the height of hyperglycaemia. Like nucleotide regeneration (Threlfall and Stoner, 1957) it did not occur if ischaemia was prolonged for 6 hr. The glycogen is probably confined to those fibres capable of recovery (Moore, Ruska and Copenhaver, 1956). Although the very low intracellular glycogen level in the muscle fibres after ischaemia and the high extracellular glucose level would favour glycogen synthesis it is difficult to see why it should differ from the rest of the skeletal glycogen in its sensitivity to adrenaline unless the mechanism whereby adrenaline activates phosphorylase has been damaged during the ischaemia. According to Kovách, Takács, Menyhárt, Iranyi and Kalmár (1952) the phosphorylation of glycogen *in vitro* by minced muscle taken from the post-ischaemic limb is inhibited.

The increased loss of liver glycogen immediately after removal of the tourniquets is not due to the action of adrenaline nor, in the rat, would one expect it to be (Sherlock, 1949). It is also unlikely to be due to hepatic anoxia (Stoner, 1958). The mechanism of this is obscure and the possibility that it reflects changes in the electrolyte composition of the liver (Hastings, Ashmore and Cahill, 1956) remains to be explored.

Haist and Hamilton (1944) considered that there was a generalized failure in the ability to store glycogen after fatal limb ischaemia. Their clear-cut results were probably due to the severe injury they used, 12–15 hr. bilateral hind-limb ischaemia. With a shorter, but usually fatal, period of ischaemia this was not an invariable feature of ischaemic injury particularly during the early part of the response when glycogen is still being formed from glucose in the muscle and liver (Table IV).

A possible increase in the formation of glucose from non-carbohydrate sources cannot be excluded. However, the glycogen lost by the liver and uninjured muscle in addition to the lactate and pyruvate released from the damaged limbs when the tourniquets are removed is sufficient to account for the increase in extracellular glucose and glycogen content of the damaged muscle during the first 3 hr. after the ischaemic period even allowing for the cost of converting the lactate and pyruvate to glucose and for the consumption of glucose at the same rate as in the normal rat (Table VI). After the first 3 hr. none of the compartments shows any increase in glucose content so that after removal of the tourniquets in the fed rat it is never necessary to postulate increased gluconeogenesis. The picture is the same in the fasted rat (Table VII). While this almost certainly takes too static a view of carbohydrate metabolism it shows that more stringent tests must be applied if increased gluconeogenesis after this injury is to be established.

Increased gluconeogenesis has usually been postulated because of the effect of injury on the adrenal cortex. The results in the fed medullectomized rats (Table VIII) are of interest here since such rats have adequate amounts of cortical

tissue and can maintain their blood sugar level on fasting (Dexter and Stoner, 1952). They should correspond to the adrenalectomized rats maintained on cortical hormone in the experiments of Selye and Dosne (1941) and Engel and Fredericks (1957) and, in accordance with the " permissive " theory of adrenal cortical action (Ingle, 1951), increased gluconeogenesis should be seen after injury. The absence of any increase in extracellular glucose in the medullectomized rat after injury shows that the normal hyperglycaemia does not depend on a " permissive atmosphere " of cortical hormone or simply on an adequate level of liver glycogen (Engel and Fredericks, 1957) and emphasizes the causative role of adrenaline.

The arguments of the last two paragraphs depend on glucose consumption during this period not exceeding that in the controls. This seems to be the case (Tables VI, VII) but the same picture would be seen if consumption was greatly increased after injury and there was a compensatory increase in gluconeogenesis. This seems unlikely. Oxygen consumption is reduced (Stoner, 1958) so that if glucose catabolism was increased the accumulation of metabolites such as lactate and pyruvate would be seen but is not (Stoner *et al.*, 1952). Although the rate of disappearance of " total body glucose " at this stage is the same as in the controls even this may be too fast, for their body temperature may be as much as 5° below that of the controls and the Q_{10} for glycolysis is 1·47–1·63 (Bendall, 1951 ; Long, 1951).

After limb ischaemia II

The terminal phase in the rat sets in 3–6 hr. after the injury and is characterized by an increasingly rapid disappearance of the " total body glucose ". Its onset corresponds to the failure of O_2 transport and the changes can be largely attributed to the decreasing supply of O_2 to the tissues.

Adrenal medullectomy and especially adrenalectomy hasten the onset of this terminal phase. This can be interpreted in two ways.

In keeping with Cannon's (1929) concept of the adrenal medulla as a gland concerned in conditioning the animal to withstand assault it might be thought that the secretion of adrenaline will mobilize the carbohydrate stores into the extracellular space where the action of the adrenal cortical hormones, by inhibiting their utilization, will preserve them for the recovery period. Adrenal medullectomy and adrenalectomy prevent these defensive manoeuvres and shorten the survival period. However, if the absence of the adrenal, wholly or in part, simply interfered with the compensatory response of the cardiovascular system to injury so that the rat was unable to maintain the O_2 supply to the tissues the same changes in carbohydrate metabolism and shortening of the survival time would occur and there would be no need to postulate effects on the enzymes of carbohydrate metabolism. The teleologically attractive scheme outlined above must be examined more critically if it is to be established.

The relation of these results to other features of the general response to injury will be discussed elsewhere (Stoner and Threlfall, unpublished). Although further problems are created, by defining the changes in glycogen distribution after injury the results go some way to answering the questions posed in the introduction and explaining the mechanism of the changes. The most important features of the results are the emphasis they put on the role of the adrenal medulla in the

causation of the changes and the questions they raise about the role of the adrenal cortex.

SUMMARY

The effect of a 4 hr. period of bilateral hind-limb ischaemia on the blood sugar level and glycogen contents of the muscle, liver, ventricles and brain was determined in fed and fasted intact rats and in fed adrenal medullectomized and adrenalectomized rats at an environmental temperature of 18–22°.

In the intact rat, during limb ischaemia there is a fall in the glycogen level in the ischaemic and uninjured muscle with a rise in the blood sugar and liver glycogen levels. These changes depended on the presence of the adrenal medulla.

During the period of adequate oxygenation after limb ischaemia, the glycogen levels in the liver and uninjured muscle of the intact rat continued to fall with a rise in the blood sugar level and some recovery in the glycogen content of the muscle in the post-ischaemic limbs. The fall in muscle glycogen level was again dependent on the presence of the adrenal medulla. The ventricular glycogen level, if submaximal, rose during limb ischaemia and for a time afterwards. Glycogen formation from glucose continued in the uninjured muscle and liver although it could not usually be stored by these tissues.

When the O_2 supply to the tissues became inadequate there was a general decline in the blood sugar and tissue glycogen levels which continued until death. This was shared by the brain where the glycogen level had remained unaltered.

No evidence for the formation of glucose from non-carbohydrate sources was found in these experiments.

The increased loss of " total body glucose " during and after limb ischaemia was attibuted, in the early stages, to the metabolic cost of transformations in the Cori cycle and in the terminal stage to anoxia.

These results emphasize the importance of the adrenal medulla in the changes in carbohydrate metabolism during and after limb ischaemia.

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ACTIVITIES OF BACTERIAL LEVANS AND OF LIPOPOLYSACCHARIDES IN THE PROCESSES OF INFLAMMATION AND INFECTION

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LEVAN and related high molecular bacterial polysaccharides given systemically to laboratory animals have been shown to modify the inflammatory response (Shilo, Wolman and Hestrin, 1954; Davies, Shilo and Hestrin, 1955; Shilo, Wolman and Wolman, 1956; Hestrin, 1956) and to lower resistance towards localised bacterial infections (Shilo, Feingold and Hestrin, 1953; Hestrin, Shilo and Feingold, 1954*a*; Hestrin, Shilo, Feingold and Wolman 1954*b*; Shilo *et al.*, 1954; Joo and Csizmas, 1957).

Likewise bacterial lipopolysaccharides have been shown to cause fluctuation of resistance of hosts towards intraperitoneal bacterial infections (Tal and Olitzki, 1948; Landy and Pillemer, 1956; Dubos and Schaedler, 1956; Boehme and Dubos, 1958) and to suppress local manifestations of acute inflammation in the dermis (Miles and Niven, 1950; Delauney, Delauney and Lebrun, 1947). It has further been demonstrated that some native levans and dextrans, in common with a number of bacterial lipopolysaccharides, interact with the properdin system *in vitro* (Pillemer, Schoenberg, Blum and Wurz, 1955) and cause a characteristic rise in the plasma properdin level *in vivo* associated with markedly increased resistance of hosts to infection (Pillemer *et al.*, 1955; Landy, 1956; Kiser, Lindh and de Mello, 1956).

Since trace amounts of lipopolysaccharides are sufficient to induce the above-mentioned biological activities and since their widespread occurrence in bacterial cells and in animal and plant tissue has been demonstrated (Landy and Shear, 1957) the question has arisen whether lipopolysaccharide contamination can account for some or all of the previously reported biological activites of neutral polysaccharides.

The purpose of the present work was to clarify which biological activities are inherent to the levan molecule and which are due to contaminating substances. The biological activites investigated were infection-promoting activity (IPA), endothelium-sealing activity (ESA), the suppressor activity on acute inflammation, necrosis-promoting activity (NPA) and the resistance-increasing activity (RIA).

METHODS

Native levans.—For the production of native levans the following strains were used: *Aerobacter levanicum*, isolated by Aschner, Avineri-Shapiro and Hestrin (1942), *Pseudomonas denitrolevaniformans* kindly given to us by Dr. A. Fuchs, Delft, and a *Corynebacterium* sp. isolated by Henis and Aschner (1954). *Aero. levanicum* levan and *Ps. denitrolevaniformans* levan were obtained from sucrose cultures and were purified by ethanol precipitation at

neutral pH as described by Hestrin *et al.* (1954*a*). *Corynebacterium* sp. levan was obtained by the action of resting cells on buffered sucrose solution as described by Avigad and Feingold (1957).

Treatment of levans with alkali. Alkaline ethanol treatment.—Native levans were precipitated from a ~ 3 per cent aqueous solution by addition of 2 volumes of alkaline ethanol, brought to pH 12 by addition of 10 N NaOH. The precipitate was resuspended in distilled water and reprecipitated in 2 volumes of alkaline ethanol. The white flocculate precipitate was dissolved in distilled water and dialysed against distilled water in the cold (4°) until pH 7 was reached (24–48 hr.). The levan was then precipitated by addition of 3 volumes of 96 per cent ethanol to 1 vol. of the solution and the gummy precipitate was converted into a white powder by persistent trituration under absolute ethanol. The powder was washed with petroleum-ether on a Buchner funnel and dried over calcium chloride.

Precipitation of levan by cetyltrimethylammonium bromide (CETAB).—A convenient method of purifying levan was afforded by the use of CETAB as a precipitating reagent in the presence of alkaline aqueous borate solution according to the principle proposed by Palmstierna, Scott and Gardell (1957) and worked out for levan in this laboratory by G. Avigad (1958). The precipitated levan was freed of salts by washing successively in aqueous methanol and petroleum ether.

Estimation of levan concentration.—The concentration of levan solutions was estimated by determining total fructose using the method of Roe, Epstein and Goldstein (1949).

Native Leuconostoc mesenteroides dextran.—Lot N-378, kindly supplied by Dr. H. Staveley, Commercial Solvents Corporation, Terre Haute, U.S.A.

Lipopolysaccharides.—AE 1688, S_4 derived from *Salmonella abortus equi* and Co08, 1684, S_4 derived from *Bacterium coli* were highly purified samples (Westphal and Luederitz, 1954) and kindly sent to us by Dr. O Westphal, Wander Forschungsinstitut, Freiburg, Germany.

In certain experiments, lipopolysaccharide was subjected to alkaline ethanol treatment for the purification of levans.

Polyvinylpyrrolidone.—Type NP (K90) kindly supplied by Mr. R. D. Goghill, Director of Research of General Aniline & Film Corporation, New York, U.S.A.

Mild acid hydrolysis of polymers.—Mild acid hydrolysis of polymers was carried out in aqueous solution at pH 3·2 (by addition of 1·0 N HCl) and heating for 60 min. at 60°. The pH of the hydrolysed solution was brought to 7 and sodium chloride added to make an 0·85 per cent solution.

Preparation of solutions and their injection.—Solutions of polymers were prepared for injection as previously described (Shilo *et al.*, 1956). Special care was taken to avoid lipopolysaccharide contamination of glassware and syringes.

Bacterial strains used in experimental infections.—*Salmonella typhi* 0901, *Salmonella paratyphi* B, strain 29 KA, *Salmonella paratyphi* C, strain 13 KA were grown in nutrient broth (Difco) for 24 hr. at 37°. *Pseudomonas aeruginosa*, strain S 64 and *Proteus* X 19 were grown on slants of nutrient agar (Difco) for 24 hr. at 37°. *Corynebacterium diphtheriae gravis* was grown on slants of brain heart infusion agar (Difco) for 24 hr. at 37°.

The above listed strains were obtained from the culture collection of the Department of Bacteriology of the Hebrew University-Hadassah Medical School.

Staphylococcus aureus, strains 9 and 33 were grown on slants of nutrient agar (Difco) for 24 hr. at 37°. These strains were isolated from cases of mastitis in sheep by Dr. T. Dishon and kindly put at our disposal.

Animals.—Albino mice 16–20 g. and adult rabbits 1½–2 kg. were used. The skin of the abdomen of the rabbits was depilated with barium sulphide before the intradermal injection of the bacteria.

Test of toxicity.—Toxicity of a given polymer was tested by intravenous injections into rabbits and mice. Diarrhoea in rabbits and mortality in mice served as an indicator of toxicity. Groups of 10 mice were used for the determination of the minimal lethal dose (MLD) of each polymer dose.

Measurement of infection-promoting activity (IPA).—IPA of a polysaccharide was tested by injecting different amounts of the polymer intravenously into mice which were infected intraperitoneally promptly thereafter with a standard sub-

lethal dose of *Salm. typhi* 0901 (1×10^7). The technique was similar to that used in our earlier work (Hestrin *et al.*, 1954*a*). The minimal amount of polymer which caused at least 80 per cent mortality served as a measure of the activity of the polymer. Groups of 10 mice were used at each test level.

Measurement of resistance-increasing activity (*RIA*).—RIA of a given polymer was tested in mice exposed to levan-enhanced *Salm. typhi* 0901 infection. The polymer to be tested was given intravenously and 24 hr. later the mice were given 10 mg. of alkaline ethanol-treated *Aero. levanicum* levan intravenously and a standard dose of *Salm. typhi* 0901 intraperitoneally. RIA was measured as the minimal amount of polymer given 24 hr. prior to the lethal challenge which saved at least 80 per cent of the infected mice. Groups of 10 mice were used at each tested polymer dose.

Test of endothelium-sealing activity (*ESA*).—Rabbits received polymer intravenously 15 min. prior to an intravenous injection of a 1 per cent solution of trypan blue (C.I. No. 437, National Aniline Division, New York) in saline. The delay in the appearance of a blue coloration at sites of irritation in the skin served as a measure of ESA. A dose of 0·002 ml. turpentine (commercial preparation) given intradermally into the depilated abdomen with the aid of an Agla microsyringe 3 min. before the administration of polymer served for producing the standard irritation of the skin. Non-levanised animals given irritant and trypan blue served as controls.

Test of suppressor activity of polymer on acute inflammation.—Polymer was injected intravenously into rabbits together with an intradermal standard dose of *Staph. aureus*, strain 9 (3×10^6 organisms) as described by Shilo *et al.* (1956). The suppressor effect of polymer on the acute inflammatory response was judged both on the basis of the induced delay of the appearance of macroscopic signs of acute inflammation (oedema and erythema) and of histologically-detectable diapedesis. Diapedesis was examined in histological sections fixed in Carnoy's fluid and stained with haematoxylin and eosin and Gram's stain. Non-levanised rabbits injected with a standard dose of staphylococci served as controls.

Measurement of necrosis promoting activity (*NPA*) *of polymer in staphylococcal lesions in the skin.*—Polymer was injected intravenously into rabbits simultaneously with an intradermal standard dose of *Staph. aureus*, strain 9 (3×10^6 organisms) as described by Shilo *et al.* (1956). The time of appearance and the size of the area of necrosis were regarded as measures of activity. Lesions were traced on cellophane paper and their areas were measured with the aid of a planimeter.

RESULTS

All tested preparations of native levan irrespective of source manifested IPA (Table II). On the other hand, only those levan preparations which had been obtained from sucrose cultures of Gram-negative organisms (*Aero. levanicum* and *Ps. denitrolevaniformans*) and which had been treated only by ethanol precipitation at a neutral pH manifested RIA (Tables I and II). The latter activity is one which such levan preparations share with a pure lipopolysaccharide preparation (Tables I and II).

The effect of mild acid treatment and of alkali on the RIA and IPA of preparations of levan and lipopolysaccharide are shown in Tables I and II. The RIA of these levan preparations was not destroyed by partial degradation of the macro-

TABLE I.—*Resistance-increasing Activity of Polymer Preparations in Respect to Levan-enhanced* Salm. typhi *Infection in Mice*

Polymer		Treatment		Resistance-increasing activity observed at different polymer doses (mg.)						
Kind	Source	A*	H†	0·001	0·010	0·05	1·0	5·0	10·0	30·0
Levan	*Aero. levanicum*	−	−	..	9/20	18/20	19/20	..	..	..
		−	+	..	..	9/10	8/10	..	..	..
		+	−	..	..	..	..	0/20	2/20	1/10
		+	+	..	..	..	..	..	..	0/20
	Ps. denitro-levaniformans	−	−	..	5/10	8/10	9/10	..	..	..
		+	−	..	..	..	..	..	0/10	..
		−	+	..	..	7/10	9/10	..	..	..
		+‡	+	..	..	..	..	..	..	0/8
	Corynebacter sp.	−	−	..	..	..	..	..	..	0/10
		−	+	..	..	..	..	..	..	0/10
Dextran	*Ln. mesenteroides*	−	−	..	..	..	..	..	..	0/20
Lipopoly-saccharide	*Salm. abortus equi* (AE 1688 S_4)	−	−	7/10	20/20	19/20	..	..	..	..
		−	+	7/9	8/10	9/10	..	..	..	..
		+	−	..	..	2/10	..	..	..	..
	Bact. coli (Co08 S_5)	−	−	6/10	19/20	10/10	..	..	..	..
Polyvinylpyrrolidone (K 90)		−	−	..	..	..	..	..	..	0/20

Resistance-increasing activity is expressed as number of mice (numerator of fraction) among those tested (denominator of fraction) which survived challenge (levan-enhanced *Salm. typhi* infection) delivered 24 hr. after injection of the specified dose of the polymer.

* Alkaline ethanol treatment.
† Mild acid hydrolysis.
‡ Alkaline CETAB treatment.

TABLE II.—*Comparison of Toxicity, Resistance-increasing Activity and Infection-promoting Activity of Polymer Preparations in Mice*

Polymer		Treatment		Minimum effective dosage[1] (mg.)		
Kind	Source	A*	H†	Toxicity	Resistance increase	Infection promotion
Levan	*Aero. levanicum*	−	−	10	0·1	5
		−	+	10	0·1	×
		+	−	×	×	5
		+	+	×	×	×
	Ps. denitrolevaniformans	−	−	5	0·1	1
		−	+	..	0·1	..
		+	−	×	10	5
		+‡	−	×	×	5
	Corynebacter sp.	−	−	×	×	2
		−	+	×	×	×
		+	−	×	×	2
Lipopolysaccharide	*Salm. abortus equi* (AE 1688 S_4)	−	−	0·1	0·001	××
		−	+	0·1	0·001	××

[1] See methods.
*, †, ‡ See Table I.

× No effect with max. dose tested (30 mg.).
×× No effect up to toxic dose (0·1 mg.).

molecule on mild acid hydrolysis. IPA, on the other hand, is known to be destroyed by such treatment (Hestrin *et al.*, 1954*a*), a feature which has been confirmed by the present experiments and extended to all the examined levans. Comparison of IPA of the different alkali-treated levans showed them to be of a similar order, *Corynebacterium* levan having highest activity. Preparations of levan which showed RIA and exerted toxic effect in animals were rendered non-toxic towards mice and rabbits and lost their RIA (Table II) by repeated precipitations with alkaline ethanol. Similar results were obtained with *Pseudomonas* levan treated with alkaline CETAB (Table II). RIA of lipopolysaccharide preparations was similarly abolished by contact with alkali in aqueous solution but was not destroyed by acid treatment which sufficed to degrade levan partially (Table I). Thus it is evident that RIA and IPA in a levan preparation were separable functions.

The IPA of alkali-treated levan in respect to intraperitoneal infections produced by several unrelated species of bacteria was evaluated in experiments summarised in Table III. It can be seen that IPA is not limited to *Salm. typhi* infections but extends also to infections produced by several other Gram-negative and Gram-positive organisms.

TABLE III.—*Infection-promoting Activity of Alkali Treated Levan Towards Intraperitoneal Infections in Mice with Different Bacterial Species*

Bacteria[1]	Mortality[2] within 48 hr.	
	With levan[3]	Without levan
Salm. typhi 0901	10/10	0/10
Salm. paratyphi B	9/10	0/10
Salm. paratyphi C.	6/10	0/10
Ps. aeruginosa	9/10	0/10
Pr. X 19	10/10	1/10
Bact. coli, 104	4/10	1/10
Staph. aureus, 9	10/10	0/10
Staph. aureus, 33	10/10	0/10

[1] Highest number of organisms which caused no mortality in absence of levan.
[2] Number of mice dying (numerator) out of those challenged (denominator).
[3] 10 mg. per mouse i/v at time of infection.

Endothelial-sealing-activity (ESA) of all alkali treated levans was clearly demonstrated by the trypan blue test. In every case the escape of trypan blue into sites of skin irritation was retarded for 30 min. or more as compared with the nearly immediate (5 min.) blueing in the non-levanised control animals, thus confirming and extending the effect described by Davies *et al.* (1955) for *Aerobacter* levans purified by precipitation at neutral pH. The suppressor activity on acute inflammation, necrosis-promoting-activity (NPA) and ESA of different levan preparations as well as dextran, PVP and lipopolysaccharides were compared using methods described by us previously (Shilo *et al.*, 1956; Davies *et al.*, 1955) (Table IV and Fig. 1). Macroscopically, suppressor activity on acute inflammation and NPA were exhibited by all the native levans tested and by the acid hydrolysates of *Aerobacter* and *Pseudomonas* levans not treated by alkali. Hydrolysates of *Aerobacter* and *Pseudomonas* levans treated by alkali and of untreated *Corynebacterium* levan were devoid of these activites.

TABLE IV.—*Effect of Alkali Treatment and Mild Acid Hydrolysis on ESA and NPA of Polymers in* Staphylococcus *Infection*

Polymer		Treatment		Quantity	Suppression of acute inflammation			
Kind	Source	A*	H†	mg./kg.	Erythema[1]	Diapedesis[2]	NPA[3]	ESA[4]
Levan	*Aero. levanicum*	−	−	100	−	−−	+	+
		−	+	100	−	−−	+	−
		+	−	100	−	−	+	+
		+	+	100	+	+	−	−
	Corynebacterium sp.	−	−	100	−	..	+	+
		−	+	100	+	..	−	−
		+	−	100	−	..	+	+
Lipopolysaccharide	*Salm. abortus equi* AE 1688 S_4	−	−	0·02	−	−−	+	..
		−	+	0·02	−	..	+	..
		+	−	0·02	+	..	−	..
Polyvinylpyrrolidone (K 90)		−	−	250	+	..	−	−
None		−	−	−	+	+	−	−

[1] + : Visible erythema and oedema < 3 hr. ; − : absent >8 hr.
[2] + : Marked leucocyte diapedesis < 1 hr. ; − : absent 5 hr. ; −− : absent > 8 hr.
[3] + : Dermonecrosis 7 hr. (spread to 14 − 20 sq. cm. 24 hr.) ; − : No dermonecrosis.
[4] + : No blueing > 30 min. ; − : Blueing 5–10 min.
*, † See Table I.

Histological examinations of animals given alkali treated *Aerobacter* levan showed no diapedesis even after 5 hr. although marked leucostasis was evident already 1 hr. after infection. In rabbits injected intravenously with hydrolysates of alkali treated *Aerobacter* levan significant diapedesis of leucocytes was found already 1 hr. after infection, phlegmonous infiltration after 3 hr., and formation of minute abscesses was evident after 5 hr.

Intravenous injection into rabbits of minute amounts (5–20 μg.) of the purified lipopolysaccharide resulted in suppression of oedema, erythema and diapedesis for 8 hr. or more. These results confirmed and extend the findings of Delauney *et al.* (1947) and Miles and Niven (1950) obtained with cruder endotoxin preparations.

The strong suppressor activity on acute inflammation and NPA serve as a delicate test for lipopolysaccharide contamination in levan preparations, provided the levan is first depolymerised by mild acid hydrolysis. In our test system clear effects were obtained by the injection of 5 μg. of purified lipopolysaccharide/kg. body weight. The presence of 5 μg. of lipopolysaccharide in a 100 mg. dose of levan, *i.e.* 0·005 per cent, could easily be detected.

In the levanised rabbit, suppression of the inflammatory response and NPA have also been shown to occur in dermal infections with *Ps. aeruginosa*, *Pr.* X 19 and *C. diphtheriae gravis* (Table V).

DISCUSSION

Treatment of levan from Gram-negative organisms with alkaline ethanol or CETAB at alkaline pH removes resistance-increasing activity (RIA) and toxicity but not infection-promoting activity (IPA), endothelium-sealing activity (ESA),

TABLE V.—*Effect of Levan on the Induction of Necrosis in Dermal Lesions Caused by Different Bacteria*

Bacteria	Levan[1]	Area of dermonecrosis and of erythema in sq. cm. with different doses of bacteria[2]		
		0·1 ml.	0·01 ml.	0·001 ml.
Staph. aureus, strain 9	+	15·0	9·5	4·0
	−	0 (3·0)	0 (1·5)	0
Ps. aeruginosa	+	20·0	9·5	2·5
	−	1·5 (3·5)	0 (1·0)	0 (0·25)
Pr. X 19	+	4·2	0	0
	−	0 (2·0)	0	0
C. diphtheriae gravis	+	34·5	0	0
	−	0 (3·0)	..	..

[1] 100 mg./kg. alkali treated *Aero. levanicum* levan 5 min. before infection.

[2] Numbers given represent the average area of dermonecrosis in two rabbits. Numbers in brackets represent the total area of lesion including erythema. Where bracketed values are not given no eythema was visible.

suppressor activity on acute inflammation and necrosis-promoting activity (NPA) from the original preparation of levan. This suggests that during the treatment some contaminating substance of the levan might have been inactivated or removed. Alkaline treatment of lipopolysaccharide is known to remove many of their biological activities (Landy and Johnson, 1955) indicating that the contaminant might be a lipopolysaccharide. The destruction of the neutral polysaccharide by mild acid hydrolysis showed that RIA and toxicity could not have been due to high molecular levan *per se* and further indicated the presence of a contaminating substance in some of the levan preparations which has both RIA and toxicity.

The lipopolysaccharide nature of the contaminant is consistent with the fact that levan produced by Gram-negative organisms rich in lipopolysaccharides contained an active contaminant whereas levan from *Corynebacter* sp. and dextran from *Leuconostoc mesenteroides*, both Gram-positive organisms known to have a low content of lipopolysaccharides, behaved as if they were initially free of this contamination.

The data obtained with purified, or initially lipopolysaccharide-free levans indicate that IPA, ESA, suppression of the inflammatory response and NPA are likely to be inherent properties of the native levan molecule, whereas RIA and toxicity, may probably be attributed to lipopolysaccharide contamination.

The previously described biological properties of *Aero. levanicum* levan and of *Ln. mesenteroides* dextran can now be further extended to other polysaccharides of the levan family of different molecular shape. *Corynebacter* sp. levan which differs from other levans in that the length of its basal chain is 1·6 times as high as that found for *Aero. levanicum* levan (Avigad and Feingold, 1957) or for *Ps. prunicola* levan (Bell and Dedonder, 1954), was shown to be similar in its biological properties to *Aerobacter* and *Pseudomonas* levans.

Although similarities between some of the biological activites of neutral polysaccharides and lipopolysaccharides are apparent, there are also important differences between the 2 groups of substances. Whereas lipopolysaccharides

act in trace amounts suggesting a trigger-like induction of a chain of reactions, often resulting in shock, neutral polysaccharides were shown not be shocking agents (Davies *et al.*, 1955) and are biologically active only when given in comparatively large amounts. It therefore seems likely that different mechanisms underlie the biological activities of the 2 groups of polysaccharides.

The findings that levan preparations are often contaminated with lipopolysaccharide makes it necessary to reconsider if some activites ascribed to levan may not have been due to a contaminant. Such a possibility may have to be considered for the following activities : the properdin-binding activity of levan *in vitro* (Pillemer *et al.*, 1955), its effect on the bactericidal activity of serum (Wardlaw, 1958), change of resistance to experimental infections (Kiser *et al.*, 1956), evocation of local Schwartzmann reaction by levan (Hestrin and Davies, 1956) as well as the effect of levan on formation of granulation tissue (Wolman and Wolman, 1956).

The use of native levan in the study of experimental infections has been hampered by the residual toxicity to animals present in some of the samples (Shilo *et al.*, 1954 ; Davies *et al.*, 1955). The proposed procedures of treatment by alkaline ethanol and alkaline CETAB make it easy to obtain non-toxic levans of high infection-promoting activity, endothelium-sealing activity and suppressor activity of acute inflammation. The levan of *Corynebacterium* sp., initially free of lipopolysaccharide activity, seems a suitable material for the study of the IPA and other biological activities of levans

SUMMARY

Native levans from *Aero. levanicum* and *Ps. denitrolevaniformans* were found to contain a contaminant showing lipopolysaccharide activity, namely resistance-increasing activity and toxicity. Treatment of these levan preparations with alkaline ethanol or alkaline cetyltrimethylammonium bromide destroyed these activities without loss of the other biological properties of the polymer, *e.g.* infection-promoting activity, endothelium-sealing activity, suppressor activity on acute inflammation and necrosis-promoting activity. Partial degradation of the levan by acid destroyed infection-promoting activity, but not resistance-increasing activity and toxicity and thus furnished further proof for a lipopolysaccharide-like contaminant. *Corynebacterium* sp. levan was found to be initially free from lipopolysaccharide activity but active in respect to promotion of infection, sealing of the endothelium, suppression of acute inflammation and promotion of necrosis in staphylococcal lesions.

The infection-promoting activity of levan towards intraperitoneal infections in mice, the inflammation modifying activity and the induction of necrosis in dermal infections in rabbits has been shown to occur with a variety of unrelated micro-organisms.

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EXPLANATION OF PLATE

Necrosis-promoting activity of *Aero. levanicum* levan preparations in staphylococcal lesions in rabbits

The rabbits received 100 mg./kg. of different *Aero. levanicum* levan preparations i.v. simultaneously with an intracutaneous injection of 3×10^6 *Staph. aureus*, strain 9 organisms.

FIG. 1: Native *Aero. levanicum* levan precipitated by ethanol at neutral pH.
FIG. 2: Same as 1 after partial degradation by acid (pH 3·2, 60 min., 60°).
FIG. 3: Native levan treated with alkaline ethanol (pH 12).
FIG. 4: Same as 3 after partial degradation by acid (pH 3·2, 60 min., 60°).
Pictures were taken 24 hr. after challenge.

Shilo and Wolman.

IN VITRO PRODUCTION OF DIPHTHERIA ANTITOXIN BY TISSUES OF IMMUNIZED ANIMALS. III. INCORPORATION OF AMINO ACIDS INTO ANTIBODY; RELATIONSHIP TO ANTIBODY SYNTHESIS AND SENSITIVITY RELATIVE TO OTHER METHODS

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In previous studies from this laboratory (Stavitsky, 1955*a*; Stavitsky and Wolf, 1958; Wolf and Stavitsky, 1958) evidence was presented for the *de novo* synthesis of diphtheria antitoxin when lymphoid tissues from immunized rabbits were incubated *in vitro* in a suitable medium. Further data obtained both *in vitro* (Stavitsky and Wolf, 1958; Wolf and Stavitsky, 1958) and *in vivo* (Taliaferro and Talmage, 1955; Stavitsky, 1955*b*, 1957*a*, 1958*a*) systems indicated that antibody synthesis required amino acids rather than more complex polypeptide or protein precursors. A synthetic medium was then developed which supported appreciable antibody synthesis as well as incorporation of radioactive amino acids into antibody by immunized lymphoid tissue *in vitro* (Wolf and Stavitsky, 1958). However, before the incorporation technique could be utilized for the investigation of aspects of antibody synthesis it was essential to ascertain whether incorporation of amino acids represented genuine synthesis of antibody. There has been much discussion (reviewed by Tarver, 1954) whether incorporation is tantamount to synthesis or whether incorporation represents exchange of protein amino acids rather than synthesis. The present study was designed primarily to determine the relationship between incorporation and antibody synthesis. Once a reasonable correlation between these phenomena was established a second purpose was to determine how sensitive a criterion of the antibody-producing capacity of a tissue was provided by the isotopic technique relative to other techniques. Finally after establishing the great sensitivity of the isotopic technique, it was applied to the preliminary study of a number of immunological phenomena which have hitherto been rather difficult to analyse.

MATERIALS AND METHODS

Animals.—The donor rabbits were male albinos which weighed 2·5–3 kg. The recipient rabbits usually also were male albinos which weighed 0·5–1 kg. They were fed Purina rabbit checkers and given water *ad libitum*.

Antigens.—The " Purogenated " alum-precipitated and fluid diphtheria toxoid and bovine γ-globulin preparations were described in a previous paper (Stavitsky, 1957*b*). The author is grateful to Lederle Laboratories for furnishing the diphtheria toxoid. In the isotopic experiments a purified diphtheria toxoid (K 39/50 kindly furnished by Dr. Inga Scheibel) was employed, which contained 2650 L_f units per mg. protein N. Ovalbumin (thrice recrystallized—Armour Laboratories) was used for the production of rabbit antisera.

* This investigation was conducted during the tenure of an Established Investigatorship from the American Heart Association.

Crystalline bovine serum albumin was purchased from Armour Laboratories. The rabbit γ-globulin was derived from an anti-pneumococcus Type I rabbit serum, generously provided by Dr. E. L. Smith of the University of Utah. The preparation and properties of this globulin were recently described (McFadden and Smith, 1955). The preparation contained 92 per cent of γ component with an S value of 6·3 and 8 per cent of a component with a value of 8·3.

Antisera.—The ovalbumin antiserum was prepared by the intravenous injection of alum-precipitated ovalbumin into rabbits as described by Vaughan and Kabat (1953). This procedure yielded consistently potent antisera containing 0·5–7·0 mg. specific antibody/ml. of serum. The bovine γ-globulin antiserum was prepared by the intravenous injection of alum-precipitated bovine γ-globulin by the same procedure as described above for ovalbumin. Rabbit antisera to diphtheria toxoid were prepared by 2 or more intravenous and foot pad injections of 16–32 L_f units alum-precipitated toxoid separated by an interval of at least 28 days and bleeding 7–8 days after the second injection. Chicken anti-rabbit γ-globulin sera were produced by the intravenous inoculation of 3·0 mg. of purified γ-globulin 4 times a week for 3 weeks followed by a bleeding 7 days after the last injection. Four antisera were prepared and tested by the quantitative precipitin procedure (Kabat and Mayer, 1948). One of these showed a single precipitin peak, clear-cut antibody excess, equivalence and antigen excess zones. Only this antiserum was used in the present study.

Antibody assay.—Antibody was measured by the haemagglutination procedure. "Purogenated" fluid diphtheria toxoid was adsorbed on sheep erythrocytes which had been treated with tannic acid according to a modification (Stavitsky, 1954*a*) of the Boyden procedure (Boyden, 1951) and the cell-toxoid complex was agglutinated by antitoxin. The potency of the antiserum was designated as the reciprocal of the highest dilution which yielded a ++ agglutination pattern (Stavitsky, 1954*a*). All the materials for serological assay from a single experiment were titrated on the same day because of the variability in titres of the same antiserum against different batches of protein-conjugated cells. All of the media employed in this study were found to be without effect upon the haemagglutination assay when a standard antiserum was mixed with the materials (1/10) and the titres of these mixtures compared with that of the same antiserum mixed similarly with saline.

In early experiments the amount of antitoxin produced *in vitro* was calculated as follows: From the sum of the reciprocal of the tissue and medium titres was subtracted the base line titre of the extract of unincubated tissue. The validity of this figure depended on the reproducibility of the weights of the tissue samples employed for homogenization and incubation. The average net weight of the lymph node fragments of a large number of samples was 14 mg. with a range of 7 to 21 mg., the average of the spleen fragments was 12 mg. with a range 5 to 20 mg. Therefore, unless the net antibody production (tissue plus medium titres minus base line titre) was at least 5 times the base line titre the experiment was disregarded.

In most of the experiments reported here the data were recalculated on the basis of 10 mg. of tissue and the net antibody production expressed as haemagglutinating units of antitoxin produced per 10 mg. of tissue. Since previously (Stavitsky, 1954*b*) a relationship had been found between haemagglutination titre and antitoxin unitage derived from biological assay, the haemagglutinating units could be expressed at least approximately in terms of units of antitoxin. Moreover, since data are available on the amount of protein per unit of antitoxin (Cohn and Pappenheimer, 1949) the titre may be expressed also in terms of quantities of antitoxic protein.

The *ring reaction* was performed in 6 × 50 mm. tubes. The antiserum was placed in the bottom of the tube and antigen carefully layered on top of the serum. The technique of Heidelberger (Kabat and Mayer, 1948) was employed with slight modification for quantitative precipitin analysis. The antigen-antibody precipitates were washed twice in ice-cold saline and the protein analysed by the Lowry method (Lowry, Rosebrough, Lewis and Randall, 1951).

Immunization and tissue culture.—Rabbits had 60 μg. (20 L_f units) of alum-precipitated diphtheria toxoid injected. Injections were made intravenously when spleens were to be cultured or into the hind foot pads when the popliteal lymph nodes were employed. When the mesenteric lymph nodes were to be utilized 4 L_f of antigen sometimes was injected directly into these nodes. Unless indicated otherwise injections were made on days 1 and 30 and organs removed for culture on day 33 since a 3-day interval between booster injections was shown to be optimal (Stavitsky and Wolf, 1958). The tissues were cut into fragments about 3 mm^3. All procedures were done aseptically. About 20 mg. of fragments (3 pieces) were placed in a 13 × 100 mm. tube containing the medium. The tubes were stoppered, placed

in a roller machine (12 rotations/hr.) and incubated for 20–24 hr. After incubation the medium was decanted and clarified by centrifugation. Since 75–90 per cent of the antibody was in the medium after incubation (Stavitsky, 1955*a*), the antibody content of the tissue at this time usually was not measured. For analysis of the tissue before incubation it was homogenized in cold physiological saline since this method was shown (Stavitsky, 1955*a*) to yield maximal amounts of antibody.

Media.—Fischer's medium (Fischer, Astrup, Ehrensvaard and Oehlenschlager, 1948), W medium (Stavitsky and Wolf, 1958), 79, 88 and 112 media (Wolf and Stavitsky, 1958) and Earle's saline (Earle, 1948) have been described previously.

Homotransplantation techniques.—Lymphoid cells were isolated by teasing the spleen or lymph nodes with the prongs at the cut edge of 60-mesh stainless steel wire mesh. One set of prongs was employed to anchor the tissue and the other to comb out the cells. The cells were suspended in Earle's saline containing 0·125 per cent gelatin. All cell transplants were made intraperitoneally. A small portion of each cell suspension was saved for analysis and the larger part transplanted. The recipient animals were untreated. Although the recipients were not treated to abolish their capacity to produce antibody against antigen in the donor cells, the antibody produced in them after transplantation appeared too rapidly (4–8 days) to be due to active immunization (Stavitsky, unpublished).

Isotope procedures.—^{35}S-L-methionine (16·3 μC/mg.) and ^{35}S yeast protein hydrolysate (about 0·58 μC/ml.) were obtained from Abbott Laboratories or Schwarz Laboratories. The hydrolysate contained radioactivity in the following precentages of labelled compounds as determined by paper chromatography : 30–50 per cent L-cystine, 20–40 per cent L-methionine and 5–15 per cent glutathione. In occasional experiments, ^{14}C DL-histidine (labelled in the 2-ring position), and ^{14}C L-leucine (1 C labelled) were employed with essentially similar results as regards specific and non-specific precipitation with the antigen-antibody aggregates.

For the determination of radioactivity of antibody the medium was centrifuged at the end of the incubation and radioactive substances, including labelled proteins which might be adsorbed non-specifically on the antigen-antibody aggregate and give a falsely high value, were removed by a heterologous antigen-antibody precipitate, as described by Keston and Katchen (1956). One to 2 mg. of egg albumin-anti-egg albumin or bovine γ-globulin-anti-bovine γ-globulin precipitates at equivalence point were employed per tube for this purpose. It was found that the formation of neither of these precipitates removed diphtheria antitoxin from the medium. Diphtheria antitoxin was precipitated by a concentration of K 39/50 toxoid which precipitated 96–99 per cent of the antibody as determined by haemagglutination titration of the supernatant. From 1–4 per cent of the antitoxin always was demonstrable in the supernatant, presumably due to agglutination of toxoid-conjugated cells by soluble antigen-antibody complexes. All of the precipitates were washed twice in cold saline containing 250 μg. of L-methionine and L-cystine per ml. and dialyzed in the cold for 2 days against 3 changes of 3 litres of saline. Visking 8/32 tubing had to be used for the dialyses because tubing of wider diameter apparently did not possess adequate surface area and dialysis in the larger tubing did not result in the attainment of constant radioactivity in 2 days. The precipitates were collected by centrifugation and resuspended in saline and dissolved in 0·25 ml. of 0·1 N NaOH. 100–750 μg. of protein were pipetted into a steel planchet, a drop of 1 per cent Aerosol OT (Eimer and Abend, New York) added, and the precipitate dried under an infra-red lamp. The radioactivity was determined with a nuclear flow counter, occasionally with a Tracerlab Autoscaler. The amount of antibody in the precipitate was calculated from the equivalence ratio of antibody to antigen for each antigen-antibody system so that the radioactivity of the immune precipitate could be recalculated in terms of the activity of the antibody itself. The observed specific radioactivity of the homologous precipitate (toxoid-antitoxin or other antigen-antibody precipitate) minus the observed radoactivity of the heterologous precipitate yielded the actual specific radioactivity of the homologous precipitate from which the specific radioactivity of the antitoxin or other antibody could be calculated.

As a check on the ability of these methods to eliminate non-specific radioactivity, it was found that toxoid-antitoxin precipitates prepared after incubation of tissues from non-immunized animals in a radioactive medium contained very little radioactivity (background or 1/3 greater).

Similar amounts of antigen-antibody precipitate were obtained when antigen and antibody were added to the various media employed in this study. It was assumed, therefore, that the medium employed did not affect the recovery of antibody.

The early isotope experiments were carried exactly like the synthesis ones with approxi-

mately 20 mg. of tissue being incubated in 1 ml. of medium with rotation in a roller assembly. The media from 3 tubes were combined for each determination. Later, however, 9 fragments (approximately 60 mg. of tissue) were incubated in 3 ml. of medium in a 20 ml. beaker in a Dubnoff metabolic shaker with gentle agitation. No striking difference in results was observed with these two methods of incubation and gentle movement of the tissues.

EXPERIMENTAL

Correlation of results of in vitro *and homotransplantation studies*

In previous studies (Stavitsky, 1955*a*) the sensitivity of the *in vitro* and homotransplantation procedures was compared by using small fragments of tissues from immunized animals for *in vitro* culture and teasing apart the larger part to provide cells for transplantation. The haemagglutination assay (Boyden, 1951; Stavitsky, 1954*a*) was used in both procedures. There was only a fair correlation between the results obtained by these 2 techniques. Occasionally the tissue produced no antibody *in vitro*, whereas isolated cells from this tissue synthesized a considerable amount of antibody upon homotransplantation. Other data in the same study (Stavitsky, 1955*a*) indicated that by both methods the antibody-producing capacity of the lymph node was maximal on the third day after antigenic restimulation of the rabbit.

The purpose of the present experiments was to obtain more definitive data regarding the relative sensitivity of the *in vitro* and homotransplantation procedures for the determination of the antibody-producing capacity of tissues in order to relate the *in vitro* activity of the tissues to an *in vivo* process. Although previous data (Roberts and Dixon, 1955; Taliaferro and Talmage, 1955; Stavitsky, 1955*a*, 1957*a* and 1958*a*; Stavitsky and Wolf, 1958) had indicated that both the *in vitro* and transplantation techniques were measuring essentially the same process of *de novo* antibody synthesis from amino acids, it was felt that more clear-cut information on this point was essential before embarking upon a study of the process of antibody formation entirely with an *in vitro* system.

Table I presents the results of a number of typical experiments. Occasionally, as noted previously (Stavitsky, 1955*a*), the homotransplantation appeared more sensitive than the *in vitro* procedure (Expts. 6, 7). But often the *in vitro* procedure appeared more sensitive (Expts. 1, 4, 8).

The formation of antibody upon transplantation of lung cells (Expt. 6) is interesting because this organ was not found active in a previous study (Stavitsky, unpublished) in which diphtheria toxoid was injected only twice. In the present experiment the toxoid was injected repeatedly. This finding confirms the results of recent experiments with egg albumin (Askonas and Humphrey, 1958) in which the lung was found to be extremely actively engaged in antibody synthesis in animals which had received many injections of antigen. The inactivity of the liver (Expt. 5) corresponds to the results of previous studies (Stavitsky, 1957*a*).

Correlation of results of in vitro *synthesis and isotopic studies*

The previous experiments had indicated that the *in vitro* synthetic procedure was at least as sensitive and often a more sensitive criterion of the antibody-producing ability of tissues than the *in vivo* homotransplantation procedure. With this information and the data (Stavitsky, 1955*a*, 1957*a* and 1958*a*; Stavitsky and Wolf, 1958) which suggested strongly that *de novo* synthesis of antibody

TABLE I.—*Relative Sensitivity of* In vitro *Culture and Homotransplantation Methods*

Expt. No.	Preparation of animal	Tissue used	Results—reciprocal titre		
			Culture	Transplant Peak	Transplant Days
1	32 Lf APT 40 mg. AP-BGG intraven. days 1, 40 and 58; tissue removed on day 61	MES	80 (Tox) 160 (BGG)	0 (Tox) 0 (BGG)	— —
2	40 mg. AP-BGG intraven. days 1 and 64; tissue removed on day 67	Spleen LLN MES	560 140 80	80 Spleens from rabbits in Expt. 2 and 3	2
3	40 mg. AP-BGG intraven. days 1 and 64; tissue removed day 67	Spleen LLN MES	1280 — —	160 LLN + MES from rabbits in Expt. 2 and 3	2
4	40 mg. AP-BGG intraven., 2·5 mg. each foot pad days 1 and 30; tissues removed day 33	Spleen LLN RLN MES	40 40 40 40	0 0 0 0	— — — —
5	32 Lf APT IV and 8 Lf APT each foot pad days 1, 5, 9, 14, 23, 26; tissues removed day 29	Liver Spleen LLN	— 20 20	— 80 80	— 3 3
6	Same as 5	Spleen Lung LLN MES	0 0 0 0	0 40 0 —	— 6 — —
7	40 mg. AP-BGG intraven., 2·5 mg. each foot pad day 1; 20 mg. BGG intraven. and 10 mg. each foot pad day 13; tissues removed day 16	Spleen LLN RLN MES	0 0 0 0	80 0 0 0	7 — — —
8	100 mg. BSA intraven. day 1 and 37; tissues removed day 40	Spleen MES	120 0	0 0	— —
9	5·6 mg. AP-EA intraven., 1·4 mg. AP-EA foot pads day 1; 1·4 mg. AP-EA intraven.; 0·7 mg. foot pads day 38; tissues removed day 41	Spleen LLN MES	40 20 10	40 0 0	4 — —

APT = alum-precipitated diphtheria toxoid; AP-BGG = alum-precipitated bovine γ-globulin; AP-EA = alum-precipitated egg albumin; LLN = left popliteal lymph node; RLN = right popliteal lymph node; MES = mesenteric lymph node.

from free amino acids was occurring *in vitro* when tissues of immunized rabbits were cultivated in a suitable medium, attention was focused upon the relationship of *in vitro* incorporation of amino acids into antibody to the actual synthesis of antibody. In a previous study (Wolf and Stavitsky, 1958) a relationship between these phenomena was suggested by the findings that the optimal concentrations of certain amino acids in the medium were required both for maximal *in vitro* incorporation as well as synthesis of antibody, and that the same W medium (Stavitsky and Wolf, 1958) supported maximal antibody synthesis as well as maximal incorporation.

Table II presents the results of a number of experiments in which the synthetic and incorporation processes were studied in parallel with the same tissue preparation and *in vitro* systems. It is clear that there was some correlation between the

2 types of data. In general a tissue which was actively synthesizing antibody, as evidenced by the haemagglutination titre of the culture medium, was also actively engaged in the incorporation of radioactive amino acids into antibody (Expts. 10, 12, 13). With increased time of incubation the extent of both processes tended to increase (Expts. 10 and 14). Usually tissues low in synthetic ability also tended to be low in incorporating capacity (Expt. 14) and a tissue lacking synthetic ability also lacked incorporating capacity (Expt. 11). However, whereas a preparation which produced antibody also incorporated amino acid into antibody, on occasion preparations, which did not seem to be synthesizing antibody according to the haemagglutination assay, were nevertheless found to be incorporating radioactive amino acid into antibody (Expts. 10 at 24 hr; 14 and 15). A discrepancy between the 2 assays was found in Expt. 13 in which there was no obvious correlation between the haemagglutination titres and the specific radioactivity of the antibody in the immune precipitates prepared from these media. This discrepancy will be discussed later.

TABLE II.—*Relative Sensitivity of* In Vitro *Culture and Isotopic Methods*

Expt. No.	Preparation of animal	Tissue	Medium	Isotope (μc. M/tube)	Hr. of incub.	Results: Culture titre	Results: Incorporation—c/m/mg. antibody: Specific	Results: Incorporation—c/m/mg. antibody: Non-specific
10	APT foot pads	LLN	88	2·5	24	160	326	147
					48	160	503	30
		RLN	88	2·5	24	0	400	186
					48	240	838	161
		Spleen	88	2·5	48	0	1,100	183
11	Ditto	LLN	W	2·5	24	0	349	349
		RLN	W	2·5	24	0	434	517
12	APT intraven.	Spleen	88	2·5	24	320	465	82
			ES			40	98	72
13	APT foot pads	LLN	W	2·5	24	80	10,800	900
			FS	—	—	93	3,100	700
			79	—	—	106	2,160	490
			88	—	—	93	1,100	860
			112	—	—	26	2,410	660
14	Ditto	RLN	W	2	20	0	106	127
					48	0	280	112
		LLN	W	2	20	0	100	104
					48	0	201	131
15	AP-EA intraven.	POP	W	0·7	24	0	12,000	32,700
		MES	—	—	—	0	14,000	24,600
		Spleen	—	—	—	0	11,200	24,300

M = ^{35}S L-methionine; H = ^{35}S yeast protein hydrolysate; foot pads = hind pads; intraven. = intravenously; ES = Earle's Saline; FS = 20 per cent rabbit serum + 80 per cent Fischer's medium.

Table II also presents evidence of the specificity of the isotopic assay. In every experiment the medium was treated first with a heterologous antigen-antibody system before the homologous antigen-antibody precipitate was prepared. It is clear from the data that the antibody in the homologous antigen-antibody precipitate contained many times as much specific radioactivity as that in the

heterologous antigen-antibody precipitate. In 2 experiments (12–24 hr. in ES, and 14–20 hr.) since the specific antibody did not contain more radioactivity than the heterologous antibody it was concluded that specific incorporation had not occurred.

Although the foregoing data suggested some correlation between the processes of incorporation of amino acids into antibody and antibody synthesis, this was far from absolute. Therefore, the relationship between these processes was analysed further by determining simultaneously the effect of various inhibitors on both processes. Table III indicates that there was a roughly corresponding decrease in incorporation and antibody synthesis when various inhibitiors were employed. Again, as noted in Table II, the relatively greater sensitivity of the isotopic procedure permitted the detection of apparent antibody-forming capacity in certain tissues in the absence of haemagglutinating antibody (Expts. 18 and 19). The inhibition of methionine incorporation by an analog of phenylalanine (Expt. 16) indicated that more than one amino acid was utilized for the synthesis of antibody. The requirement for energy for antibody synthesis and incorporation was shown by the inhibition of both processes under anaerobic conditions (Expt. 20). Arsenate and potassium cyanide also inhibited both processes, but inhibition was less consistently observed than with helium. The requirement for intact cells for both processes was demonstrated by inhibition of synthesis and incorporation when the tissues were homogenized or heated before incubation (Expts. 19 and 20). The results of comparing both synthesis and incorporation by isolated cells rather than fragments (Expt. 21) suggested that either a number of cells

TABLE III.—*Effect of Various Physico-chemical Factors on Antibody Synthesis and Incorporation*

Expt. No.	Tissue	Hr. of incub.	Isotope	Other factors	Results: Titre	Results: c/m/mg. antibody
16	POP	48	2·5 μc. M/tube	88	320	38,000
				ES	40	3,000
				88–pFlφal	40	9,400
17	Spleen	48	2·6 μc. M/tube	W	20	80,000
				W–F + T6 ×	0	0
18	,,	6	1 μc. H per beaker	W–37°	0	86,000
				W–5°	0	13,900
19	,,	1	2 μc. H per beaker	W	0	256
				W–Homog.	0	0
				W–60°/45′	0	50
20	POP	3	2 μc. H per beaker	W	120	7,470
				W–Helium	40	1,590
				W–Homog.	40	2,170
				W–pFlφal	80	4,000
21	Spleen	3	2 μc. H per beaker	Fragments (55 mg)	20	9,230
				Cells (400 mg.)	20	10,200
	POP	3	2 μc. H per beaker	Fragments (28 mg.)	160	14,760
				Cells (150 mg.)	—	24,300

POP = popliteal lymph node; M = ^{35}S methionine; H = ^{35}S yeast protein hydrolysate; pFlφal = 1 mg. of analogue per 1 ml. of medium; F + T 6 × = frozen and thawed 6 times; Homog. = tissue homogenized, whole homogenate incubated with isotope in medium; 60°/45′ = tissue heated at 60°/45′ before incubation; fragments (55 mg.) = 55 mg. of fragments incubated in medium; cells (400 mg.) = the cells isolated from 400 mg. of tissue were incubated.

or synthetic ability was destroyed during the process of isolation of the cells because the cells from large amounts of tissue were required to observe antibody synthesis or incorporation comparable to that obtained with smaller amounts of tissue fragments.

Further evidence of the relationship of incorporation to actual antibody synthesis was provided by the results of experiments in which a limited amount of radioactive histidine was placed in the medium. Under these conditions (Table IV) antibody of progressively decreasing specific activity was synthesized, while the total amount of radioactive antibody was increasing. The decrease in specific activity was also reflected in the decrease in the ratio of specific to non-specific radioactivity with time. Presumably, the limited amount of radioactive amino acid in the medium was diluted with endogenous non-labelled amino acid or with non-labelled amino acids liberated by the breakdown of tissue protein, resulting in progressive dilution of the radioactivity of the amino acid precursor in the medium. Such a relationship between incorporation and synthesis would be expected if the amino acid were being utilized for protein synthesis, but would be less likely if the amino acid were being exchanged. These data provide further evidence of the superior sensitivity of the isotopic technique since specific incorporation was observable after incubation of tissue for 1 hr. whereas a haemagglutination titre was not obtained until the tissues had been incubated for 6 hr.

TABLE IV.—*Specific Activity of Antibody and Total Radioactivity of Antibody Synthesized from Limiting Concentrations of ^{14}C-histidine**

Tissue	Time of incub. (hr.)	Results			
		HA titre	c/m/mg. Ab	Q†	Total activity (Titre × Sp. Act.)
Left popliteal node	1	0	3,920	30	39,200
	3	0	2,080	19·2	40,160
	6	40	7,350	24·7	29,400
	24	80	4,900	12·9	39,200
Right popliteal node	1	0	2,940	26·8	26,800
	3	0	2,300	12·6	23,000
	6	20	4,760	12·5	95,200
	24	80	2,980	6·92	238,400

* DL-histidine 0·5 μc. (2-ring ^{14}C).
† Q = ratio of specific to non-specific radioactivity.

Evidence that co-precipitated material is γ-globulin

In those experiments in which haemagglutinating antibody was not detected in the medium it appeared desirable to establish with greater certainty that the co-precipitated material was antibody γ-globulin. It was found that dialysis of the medium and precipitation of the γ-globulin from the medium by the method of Eisen and Pressman (1950) resulted in concentration of the co-precipitable material in the globulin rather than albumin fraction. Pre-treatment of the medium with rabbit γ-globulin- chicken anti-rabbit γ-globulin precipitates resulted in considerable diminution of the amount of radioactivity subsequently precipitated by the specific antigen-antibody system and thus provided further evidence that the co-precipitated material was γ-globulin.

Observations of various aspects on antibody synthesis by isotopic method

The preceding data indicated that the isotopic method provided a very sensitive criterion of the occurrence of antibody synthesis. This method was, therefore, applied to the preliminary re-examination of a number of aspects of the problem of antibody synthesis as well as to the exploration of new aspects of the problem. Table V provides further evidence of the superior sensitivity of the isotopic method by comparison with other methods. An apparent genetic block in the synthesis of a distinct species of antibody (anti-egg albumin) by a rabbit, as evidenced by a lack of demonstrable circulating antibody, was accompanied by an inability of its tissues to produce either haemagglutinating antibody or to incorporate amino acids into antibody (Expt. 22). Whereas it was previously believed (Stavitsky, 1955*a*) that the injection of diphtheria toxoid into the left rear foot pad did not stimulate antibody synthesis by the right popliteal lymph node, the incorporation technique indicated that the right node was active (Expt. 23). The activity of the spleen following injection of antigen into the foot pads (Stavitsky, 1955*a*, 1957*a* and 1957*b*) was confirmed by this method (Expt. 24).

TABLE V.—*Application of Isotopic Procedure to Study of a Variety of Immunological Phenomena*

Expt. No.	Preparation of animal	Tissue	Isotope	Other factors	Hr. of incub.	Results: c/m/mg. antibody: Titre	Specific	Non-specific
22	4 weekly courses of AP-EA intraven.: tissues out 7 days after last injection§	POP	0·7 μc. M/tube	—	48	0	12,000	32,700*
		MES	—	—	—	0	14,400	24,600
		Spleen	—	—	—	0	11,200	24,300
23	APT left foot pad on days 1 and 30; RLN out day 33	RLN	2·5 μc. H/tube	—	24	10	11,390	2,280†
24	APT foot pads days 1 and 30; tissues out day 33	Spleen	2 μc. H/beaker	—	48	0	1,100	183†
		LLN	—	—	—	160	500	30
		RLN	—	—	—	180	838	161
25	APT foot pads day; tissue out day 365	LLN	2·5 μc. H/beaker	W	3	—	104,200	—
				W 60°/45′	—	—	29,200	—
26	APT 32 Lf each foot pad day 1; 4 Lf left pad, 40 Lf right pad day 300; tissues out on day 303	LLN	2 μc. H/beaker	—	1	0	3,920	130‡
		RLN	—	—	1	0	2,940	110
27	APT intraven. and foot pads days 1, 42, 50; tissues out day 58	Spleen	2 μc. H/beaker	—	24	0	6,900	—
		POP	—	—	—	0	10,100	—
		MES	—	—	—	0	8,650	—

M = ^{35}S L-methionine; H = ^{35}S yeast protein hydrolysate; 60°/45′ = tissues heated at 60°/45 min. before incubation in medium.

§ No antibody found by ring reaction or quantitative precipitin reaction.

* Toxoid-antitoxin precipitate used as heterologous system.

† EA-Anti-EA used as heterologous system.

‡ BGG-Anti-BGG used as heterologous system.

Antibody synthesis by the mesenteric lymph nodes, which were difficult to stimulate except by injections of antigen directly into this organ or by the intravenous inoculation of large amounts of soluble antigen (Stavitsky, 1957*a* and 1957*b*), was demonstrated readily by the isotopic method employing the tissues of animals which had received alum-precipitated antigen intravenously and into the foot pads (Expt. 27). Employing the isotopic technique it was possible to demonstrate antibody synthesis by a variety of organs 8 days after the booster injection when haemagglutinating antibody was not demonstrable.

DISCUSSION

Although there is good evidence that the incorporation of radioactive amino acids into antibody by the intact immunized animal represents antibody synthesis (Taliaferro and Taliaferro, 1957), there appeared to be a need for more definitive evidence regarding the identity of these 2 processes as studied *in vitro*. Keston and Katchen (1956) showed that there was considerable non-specific adsorption of radioactivity in *in vitro* antibody-forming systems. The finding that the synthesis of antibody *in vitro* represented *de novo* synthesis of this protein probably identical to the process in the intact animal (see Stavitsky, 1957*a* and Stavitsky and Wolf, 1958) presented the possibility for this study of the relationship of incorporation to synthesis.

The present data indicate that *in vitro* antibody synthesis as measured by the haemagglutination method is at least as sensitive and often a more sensitive indicator of the antibody-producing capacity of tissues of immunized animals than the homotransplantation of cells from these tissues. Of course, the sensitivity of both procedures depends both upon the amounts of tissue utilized and the sensitivity of the assay for antibody.

At least 2 types of evidence are required before it can be concluded that incorporation of amino acids into antibody is related to antibody synthesis. First, it must be ascertained that incorporation is an active process which requires energy, amino acids and, perhaps, intact cells. Secondly, the radioactive material which is measured must be shown to be identical with antibody. The first of these criteria has been fulfilled by previous studies (Ogata *et al.*, 1956; Askonas and Humphrey, 1958; Wolf and Stavitsky, 1958) as well as by the present data. The second criterion has been fulfilled to some extent since the radioactive material which is measured is: (1) associated with the γ-globulin fraction of protein; (2) co-precipitated by the specific precipitate from a medium in which lymphoid tissues of immunized animals have been incubated, but not from a medium in which non-immunized lymphoid tissues or non-lymphoid tissues have been incubated; (3) not produced by the tissues of immunized animals which do not produce antibody (Expt. 22, Table V); (4) more readily demonstrable during the anamnestic response (Keston and Katchen, 1956; Askonas and Humphrey, 1958); (5) correlates roughly with antibody measured by haemagglutination; (6) produced when ^{14}C amino acids are employed so that radioactivity cannot be attributed to S-S linkage involving S-containing amino acids.

The data cited plus the failure of exchange to occur between extracellular protein and the amino acids of the medium (Askonas and Humphrey, 1958) provide strong evidence that the incorporation of amino acids into antibody is in fact a measure of genuine synthesis. It would, however, be desirable to demon-

strate the incorporation of amino acids into a peptide linkage in the antibody molecules.

There are some apparent discrepancies between the haemagglutination and isotopic assays. Some of the media (79, 88) which supported as much antibody synthesis as the W medium as measured by the haemagglutination assay sometimes supported somewhat less incorporation than on W medium. It is probably relevant that the apparent discrepancies occurred in experiments in which the tissues apparently were not synthesizing much antibody and the haemagglutination method was being employed at its lowest sensitivity whereas the isotopic method was still sensitive. In the experiments in which toxoid was employed the discrepancy between incorporation and haemagglutination may be associated to some extent with the fact that a relatively pure preparation was employed in the isotopic assay, whereas a less pure preparation was used to sensitize the erythrocytes in the haemagglutination procedure. The purity of the antigen is a less likely explanation for the other antigens, however, because the same antigenic preparations were employed in the haemagglutination and isotopic assays. A more likely explanation for the discrepancy is that some of the antibody which was measured by the isotopic method was non-precipitating. Previous studies have indicated that non-precipitating antibodies are not detectable by the haemagglutination method (Stavitsky, 1958*b*) but probably detectable by the method employed in these isotope experiments of co-precipitating any non-precipitating antibody with toxoid and precipitating antitoxin (Kuhns, 1956). There is considerable evidence suggestive of the production of non-precipitating antibody during various stages of the antibody response to various proteins (Heidelberger, Treffers and Mayer, 1940 ; Pappenheimer, 1940).

Recent evidence gives one considerable confidence in the specificity and sensitivity of the *in vitro* isotopic method for the study of antibody synthesis. In fact, it may now be stated with assurance that alterations in antibody function or the mechanisms of this process can be studied with greatest sensitivity in isolated tissues of immunized animals by the *in vitro* isotopic method. The *in vitro* method possesses other obvious advantages over *in vivo* method in that it employs less tissue, a shorter period of incubation, is subject to better control and analysis and, finally, if one biopsies part of an organ for *in vitro* study, antibody synthesis may be investigated simultaneously both *in vitro* and *in vivo*.

Among the limitations of the isotopic method as at present carried out are :

1. The small amounts of antibody produced, which may be remedied by the use of larger amounts of tissue and longer periods of incubation.

2. Rigid controls and attention to detail are needed in order to reduce the non-specific adsorption of radioactivity.

The nature of the material which is non-specifically adsorbed by the heterologous antigen-antibody precipitate is unknown. It is non-dialyzable, increases in amount with increasing time of incubation of tissue and is diminished in amount when the tissue either is damaged or incubated in the absence of amino acids (Stavitsky, unpublished). For these reasons it has provisionally been considered a protein which apparently is being synthesized by tissues under the conditions of our experiments.

With a full appreciation of its advantages and limitations it would appear that the isotopic method applied to *in vitro*-antibody-synthesizing systems is a valuable addition to the techniques applicable to the study of the basic aspects

of antibody synthesis. Preliminary attempts in this direction have been indicated in Table V. The isotopic assay has also been used successfully for the demonstration of the rapid *in vitro* induction and occurrence of both the primary and secondary antibody responses (Stavitsky, 1958*c* and *d*).

SUMMARY

Initial experiments indicated that *in vitro* antibody synthesis as measured by haemagglutination was at least as sensitive and often more sensitive a criterion of antibody-synthetic capacity of a tissue than the synthesis which occurred when cells from these tissues were transplanted to normal animals. Subsequent experiments were concerned with the relative specificity and sensitivity of the *in vitro* synthetic method compared to the isotopic assay of antibody synthesis. The isotopic assay involved the incorporation of radioactive amino acids into antibody. Evidence was obtained that incorporation was an active process requiring energy, amino acids and intact cells and, secondly, that the radioactive material produced was antibody. The data thus indicated that the incorporation method was a measure of antibody synthesis. Certain apparent discrepancies between the haemagglutination and isotopic asssays were attributed to the ability of the latter method to detect non-precipitating antibody. The material which was non-specifically adsorbed by a heterologous antigen-antibody precipitate in the isotopic assay has provisionally been considered an unknown protein which is being synthesized under the conditions of these experiments. The advantages and limitations of the isotopic method compared to other methods were discussed and it was concluded that the isotopic method is a valuable addition to the techniques used to study the basic mechanisms of antibody synthesis. Certain preliminary applications of the method were indicated.

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OBSERVATIONS ON THE INTERACTION OF POLIOVIRUS AND HOST CELLS *IN VITRO*: 1. THE EFFECT OF ENVIRONMENTAL TEMPERATURE

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DURING a study of various factors which might change the susceptibility of monkey kidney cells grown *in vitro* to polioviruses some observations were made on the effect of environmental temperature. The majority of previous observations on the cytopathic effect of polioviruses on monkey kidney epithelial cells have been made at temperatures in the range of 35–37° (Howes and Melnick, 1957; Bachtold, Bubel and Gebhardt, 1957; Darnell, 1958). The present study was concerned with temperatures outside this range.

MATERIALS AND METHODS

All experiments were made in stationary tubes (3 in. × ½ in.), in which monolayers of second-passage rhesus monkey kidney cells were grown in lactalbumin hydrolysate medium (Melnick, 1955) with the addition of 5 per cent horse serum for growth and 2 per cent horse serum for maintenance of the cultures.

After two days' growth tubes were inoculated with type I (Mahoney) poliovirus to give a final concentration of 10^3 to 10^4 TCD_{50} of virus and the tubes were then incubated at 30, 37, 39 and 41·5° in a bacteriological incubator, or at 39·5 and 40·5° in a water bath and also at room temperature (20°) and in the cold room (4°). The variation of the temperatures in the cold room and incubators was ±0·5°; the variation of temperature in the water bath was ±0·2°.

The virus titre in the tissue culture fluid at various intervals after incubation was determined by pooling the fluids from 6 to 8 tubes at the desired interval of incubation, and titrating in monkey kidney tissue culture. Cultures were examined on the 5th day after inoculation for cytopathic changes. Separate measurements of the intracellular virus were not made as it was soon realised that the amount of virus retained in the cellular debris was negligible when observations were made over a prolonged period. All the samples for any one incubation temperature were stored at −20° immediately after pooling and later titrated simultaneously. In no case were samples kept for more than three weeks at −20° before titration.

RESULTS

The amount of virus present at different times in the supernatant fluids of infected cultures incubated at the various temperatures tested is shown in Fig. 1 and 2.

In order to test the reproducibility of the titration results a comparison was made of the standard error of the mean infectivity titre when 8, 4 or 2 tubes were used for the inoculation of each dilution of virus. It was found that the standard error of the mean for 8 tubes was 0·09, for 4 tubes 0·1 and for 2 tubes

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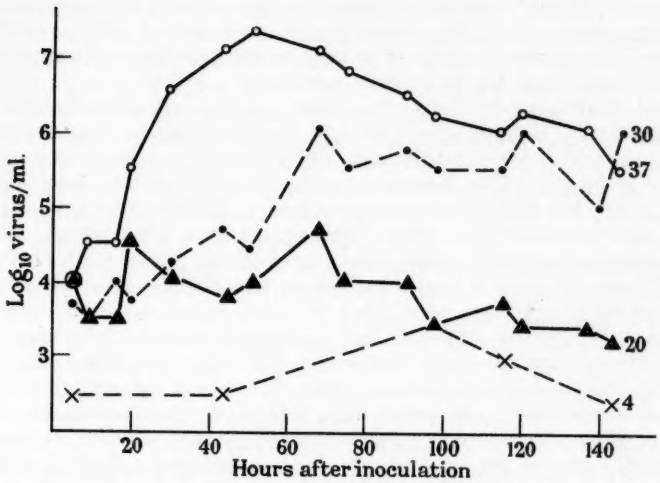


FIG. 1.—The amount of poliovirus present in supernatant fluids of cultures at given intervals of time at the temperatures shown.

Figures on the right indicate the temperature of incubation.

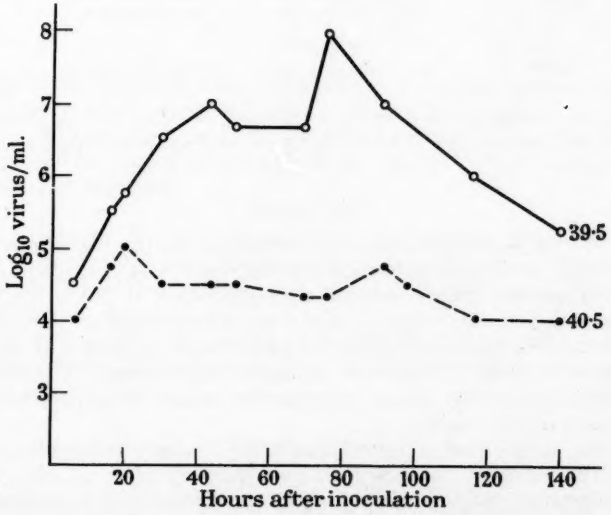


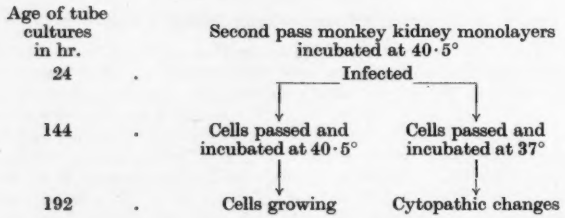
FIG. 2.—The amount of poliovirus present in supernatant fluids of cultures at given intervals of time incubated at the temperatures shown.

Figures on the right indicate the temperature of incubation.

0·23. The 95 per cent confidence limits for the titres in our experiments were therefore the mean, $\pm 0{\cdot}20$ logs for estimates made with 8 tubes, $\pm 0{\cdot}22$ logs for 4 tubes, and $\pm 0{\cdot}52$ for 2 tubes. For the majority of the results presented 4 or more tubes were used for individual titrations.

Although there was evidence of multiplication at all temperatures tested at or above 20° it will be seen (Fig. 1 and 2) that the titres increased with increase of the environmental temperature up to 39·5° at which temperature the highest titres were obtained. The increases of the viral infectivity were $10^{0.5}$, $10^{0.7}$, 10^{2}, $10^{3.2}$, $10^{3.2}$, 10^{4} and 10^{1} for temperatures 4, 20, 30, 37, 39, 39·5 and 40·5°. Although the maximal titres were obtained at 39·5°, the release of the virus at 37° was more abundant in the initial phase of the growth curve of the virus.

Of particular interest is the difference in titres obtained in tubes incubated at 39·5° and 40·5°. It was noted that in tubes incubated at 40·5° cytopathic changes did not develop. Stained cell monolayers of infected tubes incubated for 7 days at 40·5° are shown in Fig. 3, 4 and 5 and it was found that no visible difference could be detected between cells in infected tubes and control tubes held at this temperature. However, cells held at 40·5° were found to be capable of growth and to exhibit cytopathogenicity when they were incubated later at 37°. This was shown by treating the cells maintained at 40·5° with versene (0·02 per cent), and resuspending and passing them as shown below :



From the scheme above it is seen that infected monkey kidney cells incubated at 40·5° for 6 days retain the capacity to grow and show cytopathic changes.

DISCUSSION

It is well known that the temperature at which healthy plants are kept affects their susceptibility to virus infections (Kassanis, 1957) and that the environmental temperature of infected plants can determine whether or not a virus multiplies and if it does whether the signs produced are of one kind or another. Indeed the only therapeutic treatment known for plant virus diseases is to keep infected plants, or parts of them, for periods at high temperatures. Unfortunately the practical application of the same principle in animal virus infections has not given such encouraging results.

Nevertheless, it has been repeatedly reported that environmental temperature has a significant influence upon several host-virus systems. Lillie, Dyer, Armstrong and Pasternack (1937) reported the effect of seasonal temperature variation on the intensity of the brain reaction to the viruses of St. Louis encephalitis in mice and of endemic typhus in guinea-pigs. In both instances animals kept at low temperatures showed more marked reactions than those kept at high tempera-

tures. Armstrong (1942) reported that when mice were inoculated with herpes simplex virus and kept at room temperature the infection was frequently fatal, but when animals were kept at higher temperatures the majority of animals survived. A significant effect of environmental temperature was noted on the general course of experimental infection of Swiss mice infected with influenza A virus (Sulkin, 1945), animals kept at higher environmental temperatures showed significantly less pulmonary involvement than those kept at lower temperatures.

The resistance of adult mice to infections with the Conn-5 strain of Coxsackie virus is markedly lowered when mice are exposed to a cold environment during the course of infection (Walker and Boring, 1958).

The effect of induced hyperthermia on resistance of experimental animals to poliovirus infections has produced conflicting results. Jungeblutt and Kopeloff (1931) found that fever of considerable intensity induced during the period of incubation was not capable of altering the course of experimental poliomyelitis in monkeys. Wolf (1935), however, was able to abort the development of poliomyelitis in monkeys by hyperpyrexia if treatments were given soon after the inoculation and if they were continued over a long time.

From our experimental results it can be seen that in tissue culture the effect of the environmental temperature has a great influence on the poliovirus-host cell relationship. It seems that the possibilities offered by hyperthermia in poliovirus infections are not yet exhausted because in the region of 40·5° multiplication of the virus is greatly reduced and no cytopathic changes have been observed. The ability of the virus to survive in the host cells at this temperature as well as in tissue culture medium could explain the discrepancies which have been obtained in experimental animals infected with polioviruses and treated with hyperthermia.

SUMMARY

The effect of the environmental temperature on monkey kidney tissue cultures infected with type I poliovirus has been studied. The highest infectivity titres resulted from cultures kept at 39·5° but at 40·5° cytopathic changes and virus multiplication did not occur to any significant degree. The possible implications of the findings are discussed.

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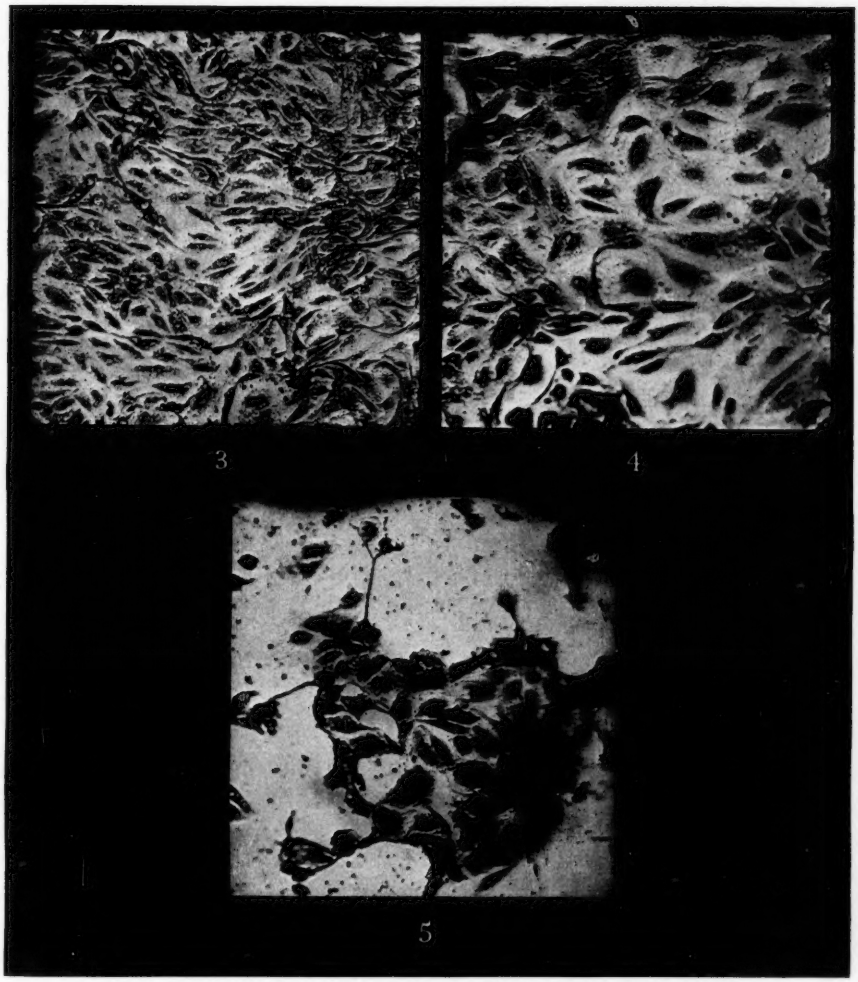
EXPLANATION OF PLATE

FIG. 3.—Normal monkey kidney cells grown at 40·5° for 24 hr. Formol saline and haematoxylin, × 240 approx.

FIG. 4.—Monkey kidney monolayers 72 hr. after inoculation with 10^4 TCD_{50} of type I poliovirus and incubation at 40·5°.

FIG. 5.—The same as in Fig. 2 after 168 hr. at 40·5°.



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GROWTH OF INFLUENZA A′ VIRUS IN CULTURED CELLS OF EMBRYO MOUSE LUNG

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THE loss of infectivity shown by influenza virus during the early stages of its multiplication has been described by Hoyle (1948) and Henle (1949).

Infective virus is, however, found in infected host cells homogenized after the inoculum has been removed. But loss of infectivity is assumed because the amount of this infective virus is very much less than the amount of infective virus taken from the inoculum by its contact with host cells. This presence of what has been called " bound infectious virus " by Ackermann, Ishida and Maassab (1955) makes it impossible to be certain that new virus arises from the greater non-infective, undetectable part of the virus in the infected cells (Ishida and Ackermann, 1956).

Evidence that new virus is derived from that part of the virus in the infected cells which becomes non-infective in the early hours after infection has been provided, in the case of herpes virus, by investigations made in this laboratory (Bedson and Gostling, 1958). Stoker (unpublished) has reached a similar conclusion. In the former work, the virus was grown in chick embryo cells in single cell dispersion in a fluid medium containing herpes antiserum, the object of the antibody being to limit, as far as possible, virus multiplication to a single cycle. Titrations of infective virus, made with disintegrated and intact cells, were compared immediately after infection of the cells, and at intervals during the early hours of incubation. The loss of infective virus observed at the 4th hour of incubation when titration was made with disintegrated cells, was no longer evident when the intact cells were used for titration.

In the present paper, experiments of somewhat similar design made with influenza virus are reported.

MATERIALS AND METHODS

The virus

The Rhodes strain of influenza A′ was used. Lyophilized material, Rhodes F4-M127 was kindly supplied by Miss Elva Minuse of the University of Michigan. This strain, isolated and passed 4 times in ferrets and 127 times in mice at Ann Arbor has been passed more than 100 times intranasally in mice since it was received here. Mice, 18–25 days old, were killed 24 hr. after intranasal infection. Their lungs were homogenized by grinding with sand and the homogenate suspended in 5 per cent inactivated normal rabbit serum (N.R.S.) in Parker's glucosol, 0·5 ml. per pair of lungs. This suspension was diluted one hundredfold in 5 per cent N.R.S. before further mice were inoculated with 0·05 ml. intranasally. Suspensions representing the 234th and 235th mouse passages of this strain were used for these experiments, either fresh or after storage at − 20°.

Preparation of cell suspensions

Balanced salt solution.—Except where otherwise stated, Hanks' balanced salt solution (B.S.S.) prepared as described by Weller, Enders, Robbins and Stoddard (1952) was used.

Trypsin.—Difco trypsin 1 : 250 made up to 0·5 per cent w/v in Hank's B.S.S. without sodium bicarbonate was stored at − 20°. Before use it was diluted with an equal volume of complete Hanks' B.S.S. and further sodium bicarbonate added to bring the pH to 7·8.

Technique.—Embryos were removed from the uteri of mice which had mated up to 20 days previously. Lungs were excised from the embryos and a cell suspension prepared from them by a technique now to be described which was based on that of Balducci, Zaiman and Tyrrell (1956). The lungs were washed twice with B.S.S., chopped with scissors into pieces approximately 1 mm. cube, and again washed in two changes of B.S.S. The lung fragments were suspended in 5 ml. trypsin in a boiling tube which was put in the 37° water bath and agitated every 2 min. After 10 min., the tube was taken from the water bath and the suspension of cells and small clumps of cells overlying the lung fragments was decanted into a centrifuge tube standing in ice water. The lung fragments were subjected to 6 further extractions with trypsin. The cells and cell clumps resulting from these last 6 extractions were deposited from the trypsin by centrifugation, and washed twice in 5 per cent N.R.S. in B.S.S.

Establishing and maintaining cultures

Chick embryo extract.—Eyes, beaks, legs and wings were removed from 9-day-old chick embryos which were washed in B.S.S. and minced with the coarsest and one but finest plungers of the Craigie mincer (Craigie, 1949). The mince was mixed with an equal volume of B.S.S. with the help of a 5 ml. syringe fitted with a lumbar puncture needle, and cleared by centrifugation at 3000 r.p.m. for 20 min. The supernatant extract was stored at − 20° and centrifugation was repeated immediately before use.

Normal rabbit serum.—Sterilization was achieved by filtration through a Ford's Sterimat (Grade SB), and the serum was heated for 30 min. at 56° before storage at 4°.

Human amniotic fluid.—Fluid was obtained from one patient in the course of rupture of the hind waters. It was sterilized by filtration through sterimats. To test for the presence of neutralizing antibodies to influenza virus, a virus suspension was titrated by simultaneous serial ten-fold dilution in broth and in the amniotic fluid; the 50 per cent egg infective dose was the same for both diluents.

Media.—" First " medium of the following composition was used :

Chick embryo extract, 8·00 per cent by volume.
Normal rabbit serum, 8·00 per cent by volume.
Human amniotic fluid, 25·00 per cent by volume.
B.S.S. 56·30 per cent by volume.
Crystalline sodium penicillin G 20,000 i.u./ml., 2·25 per cent by volume.
Streptomycin sulphate B.P. 20,000 μg./ml., 0·45 per cent by volume.

" Replacement " medium differed from " First " medium in that it contained no chick embryo extract and the concentration of normal rabbit serum was 16·00 per cent.

Cultural technique.—The washed lung cells, prepared as described above, were suspended in " First " medium and the suspension poured through 100-mesh Monel wire gauze to remove aggregates and large clumps. The suspension was distributed to insulin vials, having screw caps with siliconed rubber seals, in 2 ml. amounts. Cell counts were not made. The volume of suspension made was approximately related to the quantity of cells available. Over the course of 8 preparations the average charge put into each vial represented the cells derived from 2·03 pairs of lungs. The vials were incubated horizontally at 36·5°. Sixteen hr. later the vials were examined. If a fall in the pH of the medium and microscopy showed a satisfactory growth, the cells were used for experiment, or half the medium was discarded and 1 ml. of " Replacement " medium added to each vial, the cells being used after a further 24 hr. incubation.

Antiserum.—A neutralizing antiserum for influenza A′ virus was prepared in a rabbit. Diluted 1 : 256 in broth this serum neutralized at least 200 EID_{50} of influenza A′ virus. When diluted 1 : 16 and used as diluent for a titration of influenza infected mouse lung suspension, the titre was reduced by at least 7 $\log_{10}$ units as compared with that obtained when dilutions were made in broth.

Separation of intact infected cultured cells from vial wall

With trypsin.—In Experiment 5 the cells, having been washed 3 times with B.S.S. to remove antibody were incubated at 36·5° for 10 min. with trypsin. The vial was then centrifuged for 2 min. at 1000 r.p.m. and the trypsin discarded. The cells were washed once in B.S.S. and resuspended in 2 ml. of replacement medium. Part of the suspension was used for titration and the rest put into another vial and incubated to provide evidence of cell viability.

With versene.—In Experiments 6, 7, 8 and 9 the cells, twice washed with B.S.S. were washed a third time with phosphate buffered saline (P.B.S.) containing no calcium or magnesium (Solution " A " of the P.B.S. of Dulbecco and Vogt, 1954). Then the cells were incubated for 15 min. at 36·5° with 2 ml. of a 1 : 5000 solution of sequestric acid disodium salt (Versene) in P.B.S. (Stoker and Ross, 1958). The vial was then centrifuged and the Versene discarded. The cells were resuspended in 2 ml. of replacement medium without intermediate washing. Part of the suspension was used for titration and the remainder put into another vial and incubated to establish the viability of the cells.

Titrations.—Virus suspensions for titration were serially diluted in ten-fold steps in broth, each dilution being injected in a 0·1 ml. dose into the allantoic cavity of 4 eggs which had been incubated for 10–11 days. The eggs were examined, after 40–48 hr. further incubation, for the presence of haemagglutinin for fowl erythrocytes, and the EID_{50} computed by the method of Reed and Muench (1938).

RESULTS

Yield of virus from cultured mouse embryo lung cells

A preliminary experiment was made to establish that the cultured cells would support the growth of influenza.

Medium was discarded from 4 vials containing cells cultured for 16 hr. and replaced by 10 per cent normal rabbit serum in B.S.S. containing infected mouse lung suspension, diluted one thousand-fold. After 45 min. at 36·5° this infecting suspension was discarded and the cells washed twice with B.S.S. Replacement medium was added to the washed cells and 30 min. later a sample of this medium was removed from the vials for titration.

After 24 hr. from the first contact between cells and infecting suspension, half the medium in each vial was replaced, the part withdrawn being used for titration. A further sample was titrated at 48 hr. Results of this experiment are shown in Table I under Experiment 1. It can be seen that the infected cells were able to increase the concentration of infective virus in the medium by 3 $\log_{10}$ units in 24 hr.

TABLE I.—*Infective Influenza A′ Virus in Culture Medium of Infected and Washed Mouse Embryo Lung Cells*

Time (hr.)	EID_{50} of medium in ml.		
	Experiment 1	Experiment 2	Experiment 3
<2	$>10^{-1}$	$10^{-1.5}$	$10^{-2.7}$
2½	—	$10^{-1.3}$	—
4	—	$10^{-1.3}$	$10^{-1.7}$
5½	—	$10^{-1.2}$	—
7	—	$10^{-1.5}$	$10^{-2.3}$
9	—	—	$10^{-2.7}$
24	$10^{-4.1}$	$10^{-4.0}$	$10^{-4.5}$
48	$10^{-4.8}$	$10^{-4.2}$	$10^{-4.7}$

Time measured from first contact between cells and infecting virus suspension.
EID_{50} = 50 per cent egg infective dose.

In Experiments 2 and 3 the medium was examined in the hours following the initial sampling. Reference to Table I shows that the quantity of infective virus present in the medium at 7 hr. in Experiment 2, and 9 hr. in Experiment 3 does not exceed that found within 2 hr. of the beginning of infection, though there is a suggestion that earlier loss from thermal inactivation has begun to be made good.

Infective virus in the cells

The cells were also examined in the course of these two experiments, and in those which followed, to see whether infective virus could be found within them during the early hours after infection. For this purpose infected cells were grown in the presence of rabbit serum containing neutralizing antibodies for influenza A′. Mouse embryo lung cells grown for 16 or 40 hr. in a series of vials were infected and the infecting inoculum removed as described above. After the first sample of medium had been taken from the vials, antiserum was added to some of them 90 min. after first contact of the cells with the infecting syspension. The final concentration of antiserum in the vials was 1/60. At intervals thereafter the antibody containing medium was removed from a vial and tested by allantoic inoculation in 4 eggs to exclude the presence of infective virus. The cells were washed three times with B.S.S. and 2 ml. (the original volume of medium) of 5 per cent N.R.S. in B.S.S. added to them. With the cells submerged, the contents of the vial were frozen and thawed three times and titrated in eggs.

Egg infectivity of suspensions of intact infected cells

At the completion of Experiment 4 (see Table II) it was apparent that the quantity of infective virus found early in the growth cycle, associated with the cells in such a way as to be insusceptible to antiserum, did not influence the yield of new virus at 24 hr. So in Experiments 5, 6, 7, 8 and 9, at the same

TABLE II.—*Infective Influenza A′ Virus in Mouse Embryo Lung Cells Grown in the Presence of Antiserum to the Virus*

Material	Time (hr.)	EID_{50} in ml. of tested material, Experiment 2	3	4	5	6	7	8	9
Culture medium before adding antiserum	$<1\frac{1}{2}$	$10^{-1.5}$	$10^{-2.7}$	$10^{-3.5}$	$10^{-1.5}$	$10^{-1.6}$	$10^{-2.4}$	$10^{-2.2}$	$10^{-2.3}$
Infected cells grown in the presence of antibody, washed and disintegrated in diluent equal in volume to that of their medium	2	N.P.E.	—	—	N.P.E.	$>10^{-1}$	10^{-1}	10^{-2}	N.P.E.
	4	N.P.E.	—	N.P.E.	N.P.E.	$>10^{-1}$	N.P.E.	$>10^{-1.0}$	N.P.E.
	$4\frac{1}{2}$	—	N.P.E.	—	—	—	—	—	—
	$5\frac{1}{2}$	—	—	$10^{-1.5}$	—	—	—	—	—
	6	—	—	—	N.P.E.	10^{-1}	N.P.E.	$10^{-1.5}$	$10^{-1.0}$
	$6\frac{1}{2}$	N.P.E.	—	—	—	—	—	—	—
	7	—	—	$10^{-2.3}$	—	—	—	—	—
	$7\frac{1}{2}$	—	$10^{-1.6}$	—	—	—	—	—	—
	8	—	—	—	$>10^{-1}$	$10^{-1.2}$	N.P.E.	$10^{-2.6}$	$>10^{-1.0}$
	$8\frac{1}{2}$	—	—	$10^{-1.8}$	—	—	—	—	—
Culture medium to which no antiserum was added	24	$10^{-4.0}$	$10^{-4.5}$	$10^{-4.4}$	$10^{-5.0}$	$10^{-4.3}$	$10^{-3.5}$	$10^{-5.3}$	$10^{-4.2}$

Time measured from first contact between cells and infecting virus suspension.
EID_{50} = 50 per cent egg infective dose.
N.P.E. = No positive eggs.

time intervals as the cells in other vials were washed to remove antiserum and frozen and thawed, infected cells in some of the antiserum-treated vials were, after similar removal of antiserum, freed from the glass with trypsin or versene and resuspended to the original volume in new medium. The suspensions of dispersed cells were titrated in eggs by inoculation of serial dilutions into the allantoic cavity.

It was assumed that if the cells, at the time of dispersal, were in course of producing new virus from non-infective virus, they would survive long enough in the titration eggs for the new virus to become infective, be liberated from the cells and infect the allantois. A higher titre registered by these dispersed cells than by samples removed at the same stage of incubation and disintegrated, would indicate that the number of cells in process of virus production in the cultures was greater than the number of doses of infective virus present at the same time, and that new virus must be coming from non-infective material.

Table III sets out the results of titrations of disintegrated and dispersed cells in Experiments 5, 6, 7, 8 and 9.

TABLE III.—*Comparison of EID_{50} of Cultured Mouse Embryo Lung Cells, Infected with Influenza A′, Intact and Disintegrated*

Material	Expt.	EID_{50} in ml. of tested material							
		2 hr.		4 hr.		6 hr.		8 hr.	
		Intact	Disintegrated	Intact	Disintegrated	Intact	Disintegrated	Intact	Disintegrated
Infected cells grown in presence of antiserum to influenza and washed	5	N.P.E.	N.P.E.	$>10^{-1.0}$	N.P.E.	$>10^{-1.0}$	N.P.E.	$10^{-2.2}$	$>10^{-1.0}$
	6	$10^{-1.7}$	$>10^{-1.0}$	$10^{-1.8}$	$>10^{-1.0}$	$10^{-2.0}$	$10^{-1.0}$	$10^{-2.3}$	$10^{-1.2}$
	7	$10^{-1.5}$	$>10^{-1.0}$	$10^{-1.5}$	N.P.E.	$10^{-2.3}$	N.P.E.	$10^{-1.2}$	N.P.E.
	8	$10^{-2.5}$	$10^{-2.0}$	$10^{-1.4}$	$>10^{-1.0}$	$10^{-1.7}$	$10^{-1.5}$	$10^{-3.6}$	$10^{-3.6}$
	9	$>10^{-1.0}$	N.P.E.	$10^{-1.3}$	N.P.E.	$10^{-1.3}$	$10^{-1.0}$	$>10^{-1.0}$	$>10^{-1.0}$

Time is measured from first contact between cells and infecting virus suspension.
EID_{50} = 50 per cent egg infective dose.
N.P.E. = No positive eggs.

It can be seen that at stages of the cycle up to 8 hr. after the beginning of infection, dispersed intact cells have a higher titre than disintegrated cells in almost every case.

DISCUSSION

The term "initiating activity" was used by Ackermann *et al.* (1955) to describe virus which is successful in initiating virus increase. The fact that in the experiments recorded here (Table II) the quantity of cell-associated infective virus does not influence the yield of new virus is not, of itself, proof that such virus is not the initiating activity. Yield in the culture system here described probably depends partly on available cells, and inactivated virus in a large inoculum might, by interference, block susceptible cells so that no more than one cycle of multiplication could take place. It has been remarked by Ledinko, Bergs and Henle (1957) of a culture system resembling that employed here, namely suspended chick embryo lung cells, that the system did not lend itself to accurate quantitative analyses of cell-virus relationships. But the evidence summarized in Table III,

which shows that in the early hours of the cycle there are up to ten times as many cells producing virus as there are doses of infective virus in the cells at each sampling time, is sufficiently consistent to make it clear that, as in the case of herpes (Bedson and Gostling, 1958), initiating activity is with non-infective influenza virus.

SUMMARY

The preparation of cultures of cells separated from the lungs of embryo mice by digestion with trypsin is described.

Influenza A′ virus was found to multiply in these cultures. During the first 8 hr. after infection of the cells infective virus could be found associated with them when the medium in which they grew contained neutralizing antiserum against the virus. The quantity of infective virus found at this stage did not influence the yield of new virus found after 24 hr. incubation.

In further experiments samples of infected cultured cells were removed from incubation in a medium containing antiserum 2, 4, 6 and 8 hr. after first exposure to infection. After washing, titrations were made both of suspensions of the cells, dispersed and intact, and after disruption by freezing and thawing. At each interval the egg infective titre of intact cells was up to ten times greater than that of disrupted cells.

It was concluded that new virus found after 24 hr. must have come from virus which was non-infective in the hours immediately following infection.

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SURVEY OF PAPERS

HARRIS subjected guinea-pigs to whole body irradiation and assessed lymphocyte levels in the bone marrow, thymus gland and mesentery lymph node. Following irradiation the lymphocytes in the thymic cortex were reduced resulting in a diminution of the thymic weight and the bone marrow lymphocytes were also reduced. The lymphocytes in the bone marrow, thymus and mesenteric node returned to normal in 35 days and preceded the recovery of the myeloid elements in the bone marrow (p. 557).

MARTINEZ, JUNE SMITH and GOOD have successfully transplanted ovaries and hypophyses into immunologically tolerant, ovariectomized or hypophysectomized, genetically different recipient mice. They present evidence that the transplanted glands continued active physiological function in the recipients for long periods (p. 574).

O'GRADY reports that tuberculous mouse thigh lesions fell in size when tuberculin was injected into the other limb. When tuberculin was repeated at intervals of a few days the fall in size of the lesions continued for the first few weeks but the lesions then escaped from the therapy. Maximal response to the treatment corresponded therefore with the period of sensitization and not desensitization. Animals in which focal reactions occurred fared worse than their controls (p. 582).

SOMMERVILLE, MCINTOSH and CARSON have compared the multiplication rate and plaque production of four types of ECHO virus representing the four groups defined by other workers. Monolayers of monkey kidney epithelial cells were used and the growth cycles were very similar to those of poliovirus. There were, however, marked differences between the different types of ECHO virus in both virus and plaque production although no direct correlation existed between these two properties (p. 589).

HARRIS and MARIANNE JAHNZ have studied the incorporation of isotope-labelled amino-acids by homologous populations of rabbit macrophage and HeLa cells. No differences in the pattern of incorporation have been observed between these 2 cell-types or by comparison of the present findings with those previously reported for rat connective tissue (p. 597).

WILCOX, WOOD, JANG OH, EVERETT and EVANS have studied the effects of intraocular inoculation of Newcastle disease virus and Influenza A virus upon the corneal endothelium in rabbits. They show that both viruses produce progressive destruction of the endothelium causing an altered exchange relationship between the corneal stroma and the aqueous humour which results in corneal oedema and opacity. Regeneration of the endothelium was associated with diminution of oedema and opacity, and the development of a transitory refractoriness to challenge with the homologous virus. They failed to demonstrate virus multiplication in the cornea (p. 601).

BRUMFITT and HEPTINSTALL report that in rats injected intravenously with *Bact. coli* multiplication did not occur in the normal kidney. Permanent obstruction of one ureter resulted in progressive proliferation of organisms in that kidney. Release of the occlusion after 6 or 24 hr. caused the organism counts to fall. This does not support the view that infection is initiated in the obstructed kidney by increased deposition of organisms (p. 610).

LANDY, TRAPANI and SHEAR found the inhibition by polysaccharides of properdin inactivation of phage to be a useful method of measuring polysaccharide-properdin interaction. The anti-phage activity of properdin was annulled by polysaccharide as a linear function of dose which could be examined in the range 1–30 μg. (p. 618).

HERRING and KENT have examined mucoproteins obtained by alkaline extraction of ox tracheal tissue. Components separated and purified by electrophoresis are described (p. 631).

STONER, continuing his studies on the mechanism of shock, has related changes in carbohydrate metabolism after limb ischaemia to the adequacy of tissue oxygenation. The importance of the adrenal medulla in these changes is emphasized. Glycogen formation from glucose continued in the tissues even when glycogen could not be stored but there was no increased synthesis from non-carbohydrate sources (p. 635).

SHILO and WOLMAN found that *Corynebacterium* sp. levan showed infection promoting activity, endothelium sealing activity, suppression of acute inflammation and necrosis promotion. They present evidence that the added properties of resistance increasing activity and toxicity which native levans from *Aero. levanicum* and *Ps. denitrolevaniformans* showed are due to the presence of lipopolysaccharide contaminants (p. 652).

STAVITSKY has shown that *in vitro* antibody synthesis is at least as sensitive a criterion of the antibody synthetic capacity of a tissue as the synthesis which occurs when cells from such tissues are transplanted into normal animals. He has studied the relative specificity and sensitivity of *in vitro* synthesis compared to isotopic assay of antibody synthesis. He concludes that the isotopic method is a valuable addition to the techniques available for the study of the basic mechanisms of antibody synthesis (p. 661).

LIKAR and WILSON have incubated monkey kidney cell monolayers infected with poliovirus at various temperatures and have compared the virus yield at intervals and the development of cytopathic changes. The highest yields were from cultures held at 39·5°. Much smaller amounts of virus were obtained from cells kept at 40·5° and, although no cytopathic changes developed at this temperature, the effect became visible when the cultures were incubated later at 37° (p. 674).

GOSTLING has studied the multiplication of Influenza A′ virus in cells from trypsinized mouse embryo lungs ; immune rabbit serum was added after infection of the cells and at 2 hr. intervals up to 8 hr. the amount of virus from washed disintegrated cells compared with titrations on whole cell suspensions. At each of these intervals up to ten times more virus was recovered from the intact cells (p. 679).

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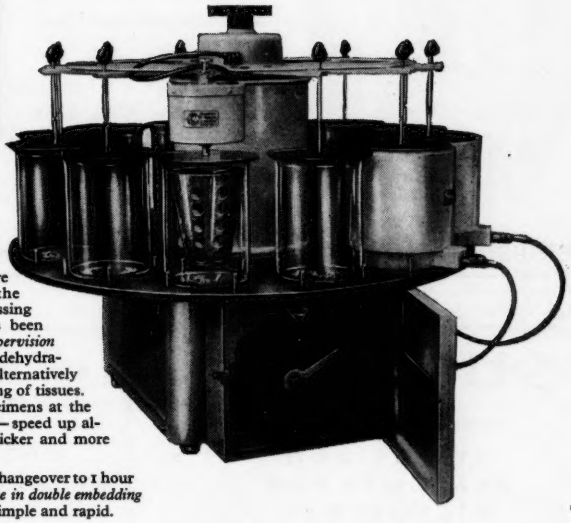

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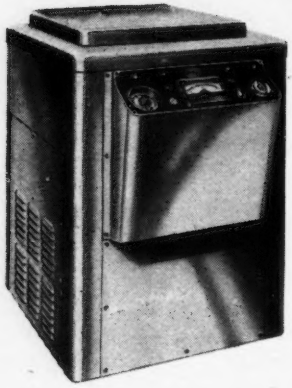

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